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INSULIN-SPARING EFFECT OF HYDROXYCHLOROQUINE IN RATS

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"Insulin-Sparing Effect of Hydroxychloroquine in Rats"** submitted by Jaber Emami Bafrani in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences.

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This thesis is dedicated to:

My parents, for their unending support, guidance and love,

I've been blessed to be the recipient of throughout my life.

My wife, Mozhgan, and my daughter, Maryam,

whose presence is a constant reminder of

support, encouragement, and hope.

ABSTRACT

Hydroxychloroquine (HCQ) is a racemic antimalarial drug increasingly used as a disease-modifying agent in rheumatoid arthritis. Attention has recently been focused on the effects of this drug on insulin and glucose metabolism in diabetes. Two studies of diabetic patients resistant to standard therapy have demonstrated significant improvement in glycemic control following HCQ therapy. We hypothesized that the effect of HCQ on serum glucose levels in diabetes is due to inhibition of insulin clearance. There is a strong correlation between this effect and HCQ blood concentrations.

Our objectives were: 1. To study the physiological manifestations of HCQ administration in the non-diabetic state. 2. To determine the effect of this drug on glycemic profiles in diabetic animals. 3. To elucidate the mechanism by which HCQ affects glucose and insulin metabolism. 4. To study the pharmacokinetics of HCQ in the presence of arthritis and diabetes, diseases amenable to HCQ therapy.

Therefore, this study was undertaken to investigate the hypoglycemic effects of HCQ in both healthy and diabetic rats and to elucidate the mechanism(s) by which this drug may exert its effects. The effect of experimental diabetes and arthritis on the disposition of HCQ enantiomers was also studied.

Findings of this study: 1. Hydroxychloroquine treatment, did not influence fasting serum glucose levels, insulin sensitivity or glucose tolerance in non-diabetic rats. 2. Significant improvement in glycemic and insulin profiles was observed in diabetic rats treated with HCQ. This effect was HCQ concentration-dependent. 3. *In vitro* intrinsic

clearance of insulin in the cytosolic fraction of rat livers of both diabetic and non-diabetic rats was significantly reduced in the presence of HCQ. 4. Diabetes caused a significant increase in renal and non-renal clearance of HCQ enantiomers whereas the distribution was mainly unaffected. Diabetes-induced effects on the disposition of HCQ were reversed by insulin treatment. 5. In arthritic rats, systemic clearance of HCQ enantiomers was significantly reduced.

In conclusion HCQ improved glycemic control in the diabetic state through inhibition of insulin metabolism. Diabetes increased the systemic clearance of both HCQ enantiomers. Adjuvant arthritis, on the other hand, caused a significant decrease in systemic clearance of HCQ enantiomers. The effect of the diseases, however, was not stereoselective as the pharmacokinetics of both enantiomers were affected almost equally.

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LIST OF SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

A	Extrapolated concentration at time zero for the distribution phase
α	Level of significance (probability of making type I error)
ANOVA	Analysis of variance
$AUC_{0-\infty}$	Area under the blood concentration time curve from zero to infinity
AUC_{0-t}	Area under the blood concentration time curve from zero to time t
AUG	Area under the percent reduction in glucose-time profile
AUI	Area under the percent increase in serum insulin-time profile
AUMC	Area under the first moment curve
β	Elimination phase rate constant
B	Extrapolated concentration at time zero for the elimination phase
B/P	Blood to plasma ratio
BSA	Bovine serum albumin
°C	Degrees Celsius
CL_r	Renal clearance
CL	Systemic clearance
Cl_i	Intrinsic clearance
Cl_{nr}	Non-renal clearance
C_{max}	Maximum (peak) blood drug concentration after single dose administration
C_s	Substrate concentration
C_t	Total drug plasma concentration (bound and unbound)
C_{uf}	Ultrafiltrate drug concentration
CV	Coefficient of variation
cm	Centimeter(s)
dL	Deciliter
F	Absolute bioavailability
FFA	Free fatty acid(s)
Fig.	Figure

F_u	Fraction unbound
GFR	Glomerular filtration rate
g	Centrifugal force
HCQ	Hydroxychloroquine
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
h	Hour(s)
IDDM	Insulin-dependent diabetes mellitus
i.d.	Inside diameter
i.v.	Intravenous
Kg	Kilogram(s)
KHB	Krebs-Henselite phosphate buffer
K_m	Michaelis constant
L	Liter
M	Molar
MHC	Major histocompatibility complex
MRG	Percent maximum reduction in serum glucose
mg	Milligram(s)
µg	Microgram(s)
µL	Microliter(s)
min	Minute(s)
mL	Milliliter(s)
NIDDM	Non-insulin-dependent diabetes mellitus
NSAID	Non-steroidal anti-inflammatory drug(s)
n	Number
ng	Nanogram(s)
nm	Nanometer(s)
o.d.	Outside diameter
P	Probability of rejecting the null hypothesis when it is true
p.o.	Peroral

r	Pearson correlation coefficient
S.D.	Standard deviation
S.E.	Standard error
ΣX_u	Cumulative urinary excretion
SLE	Systemic lupus erythematosus
STZ	Streptozotocin
s.c.	Subcutaneous
TCA	Trichloroacetic acid
T_{max}	Time to reach peak or maximum concentration after drug administration
t1/2	Elimination half-life associated with the terminal slope of a semilogarithmic concentration-time curve
U	Unit(s)
US	United States
UV	Ultraviolet
V	Degradation rate
V_{max}	Maximal reaction velocity
Vss	Volume of distribution at steady state
vs	Versus

Chapter 1

INTRODUCTION

Hydroxychloroquine (HCQ) is a chiral antimalarial agent which is also an effective disease modifying drug against rheumatoid arthritis (Pullar 1990; Clark *et al.*, 1993; The HERA study group, 1995). Attention has recently been directed towards the effects of this drug on insulin and glucose metabolism in diabetes mellitus. A literature review on the effects of HCQ on glucose homeostasis reveals a placebo controlled trial which demonstrated a significant improvement in glucose levels in patients with NIDDM resistant to standard therapy (Quatraro *et al.*, 1988). A very recent study of 135 patients also showed that HCQ significantly improved glycemic control in patients with NIDDM (Gerstein *et al.*, 1997). These trials suggest that this drug may be therapeutically useful for the treatment of type I and II diabetes mellitus.

In spite of these trials, little information is available regarding the mechanism by which HCQ affects glucose and insulin metabolism. Chloroquine, which is structurally very similar to HCQ (Fig.1-2), has been used to study intracellular insulin processing. *In vitro* studies suggest that chloroquine reduces intracellular insulin degradation, increases intracellular insulin accumulation, slows receptor recycling, and stimulates insulin-mediated glucose transport (Smith *et al.*, 1989; Cynober *et al.*, 1987).

The physiological manifestations of HCQ in non-diabetic state and the effect of this drug on glycemic profiles in animal models of diabetes need to be characterized. This seems essential to further study the mechanism of HCQ action on glucose homeostasis. In addition, the pharmacokinetics of HCQ needs to be studied in the presence of diabetes and

arthritis, diseases amenable to HCQ therapy. Therefore, this study was undertaken to investigate the hypoglycemic effects of HCQ in both healthy and diabetic rats and to elucidate the mechanism by which this drug may exert its effect. We also studied the effect of experimental diabetes and adjuvant-induced arthritis on the disposition of HCQ enantiomers.

1. DIABETES MELLITUS

Diabetes mellitus is the most common of all endocrine diseases. It is characterized by metabolic abnormalities, long term complications involving the eyes, kidneys, nerves and blood vessels, and by a lesion of the basement membranes demonstrable by electron microscopy. The following information regarding physiopathology of type I and type II diabetes was obtained from relevant text books, Internal Medicine (Kelly *et al.*, 1997) and Endocrinology (Wilson & Foster, 1997).

1.1. Diagnosis

1.1.1. Symptomatic Diabetes

Diabetes mellitus is recognized on the basis of elevated plasma glucose (abnormal glucose tolerance) and classic symptoms including polydipsia, polyuria, polyphagia and weight loss.

1.1.2. Asymptomatic Diabetes

The patient is considered to be a potential diabetic but has a normal fasting glucose concentration in plasma. Such patients demonstrate impaired glucose tolerance following an oral glucose tolerance test (2h following ingestion of 75 g glucose, serum glucose ≥

200 mg/dL). A normal glucose tolerance test is strong evidence against the presence of diabetes, however, predictive value from a positive test is less certain.

1.2. Classification

A classification of diabetes and other categories of glucose intolerance was developed by the National Diabetes Data Group in 1980. Table 1-1 summarizes the new as well as old classification system. Insulin dependence in this classification is not equivalent to insulin therapy. Rather, the term means that the patient is at risk for ketoacidosis in the absence of insulin. Many patients classified as non-insulin-dependent (20% of patients with NIDDM) require insulin for control of their hyperglycemia, although they do not become ketoacidotic if insulin is withdrawn. The terms *insulin-dependent* and *non-insulin-dependent* describe physiologic states (ketoacidosis-prone and ketoacidosis resistant, respectively) while the terms *type I* and *type II* refer to pathogenic mechanisms (immune-mediated and non-immune-mediated, respectively) of the disease.

1.3. Prevalence

Diabetes mellitus and its complications are now the third leading cause of death in the United States (US). Patients who are diagnosed with diabetes include 2.8% of the US population. A new case of diabetes is diagnosed every 60 seconds, and the chance of developing diabetes doubles with every 20% of excess weight and every decade of life. Overall, the economic cost of diabetes more than quadrupled over the 5-year period between 1987 and 1992, increasing from 20 billion dollars to 92 billion dollars. As an example of the cost of diabetic complications, approximately one-third of all cases of end-stage renal disease in the US are the result of diabetes. It is estimated that the cost of

diabetes-induced end-stage renal disease in the US is between 2 and 3 billion dollars per year (Herfindal and Gourley, 1996).

Table 1-1. Classification of diabetes mellitus.

Current terminology	Other terminology
PRIMARY DIABETES MELLITUS Type I, Insulin dependent diabetes mellitus (IDDM)	-Juvenile onset diabetes (JOD) -Youth onset diabetes (YOD) -Ketosis prone brittle
Type II, non-insulin- dependent diabetes mellitus (NIDDM) **Subtypes:** -Obese -Non-obese -Maturity onset diabetes of the young	-Adult onset diabetes (AOD) -Maturity onset diabetes (MOD) -Ketosis resistant
Mixed types	- Latent autoimmune diabetes in adults -Diabetes of young blacks
Gestational diabetes mellitus (GDM)	
Type III diabetes mellitus	-Malnutrition-related diabetes mellitus
Impaired glucose tolerance (IGT)	-Asymptomatic diabetes -Chemical diabetes -Borderline diabetes -Latent diabetes
SECONDARY DIABETES MELLITUS -Pancreatic disease: Pancreatic surgery, Chronic pancreatitis -Chronic liver disease -Endocrinopathies: Cushing's syndrome, Acromegaly, Pheochromocytoma, Aldosteronism -Medication use: Glucocorticoids, β-Blockers	

1.4. Pathogenesis of Diabetes Mellitus

1.4.1. Type I, Insulin-Dependent Diabetes Mellitus

By the time insulin-dependent diabetes mellitus (IDDM) appears, most of the β cells in the pancreas have been destroyed. The destructive process is almost certainly autoimmune in nature, although details remain obscure. A tentative overview of the pathogenic sequence is as follows: ***First***, genetic susceptibility to the disease must be present. ***Second***, an environmental event (non-genetic factors) ordinarily initiates the process in genetically susceptible individuals. The ***third*** step is activation of immune mechanisms targeted against pancreatic islet β cells. The ***fourth*** step is an alteration or transformation of the surface of the β cell such that it is no longer recognized as "self, " but is seen by the immune system as a foreign cell or "nonself." The ***fifth*** step is the development of an immune response. Because the islets are now considered "nonself," cytotoxic antibodies develop and act in concert with cell-mediated immune mechanisms. The end result is the destruction of the beta cell and the appearance of diabetes. Rarely, type I diabetes may develop from an exclusive environmental insult. It is also possible that in some cases autoimmune diabetes develops in the absence of an environmental trigger; that is, it is purely genetic.

1.4.1.1. Genetic Susceptibility

A familial predisposition to type I diabetes has long been known but a specific mode of genetic transmission has not been established. There is a higher concordance rate for type I diabetes in monozygotic twins (35% to 50%) than in dizygotic twins (5% to 10%) indicating that genetic factors are not solely responsible for the disorders. Overall,

the chance of a child developing type I diabetes, when another first-degree relative has the disease, is only 5 to 10%. The presence of NIDDM in a parent increases the risk for IDDM in the offspring. Genetic susceptibility to IDDM appears to be linked to certain human leukocyte antigen (HLA) alleles in major histocompatibility complex (MHC) on the short arm of the sixth chromosome. A number of loci designated by the letters A, B, C, and D have been firmly established with alleles at each site identified by numbers (HLA-B8 & HLA-DR3). HLA gene products (class I from A, B, C, loci and class II molecules from D region) are located in the plasma membranes of cells and are best considered as recognition and/or programming signals for initiation and amplification of immune response in the body. Class I molecules are present on all nucleated cells and function primarily in defence against infections (especially viruses). Class II molecules are normally present on circulating and tissue macrophages, endothelial cells, B lymphocytes, and activated T lymphocytes. They function in the regulatory T-cell system. They are also important in auto-immune diseases such as type I diabetes. Antigens associated with class I molecules are recognized by $CD8^+$ cytolytic T lymphocytes, whereas class II associated antigens are recognized by $CD4^+$ helper T cells. Activation of the immune system is MHC restricted which means that antigens are recognized only if they reach the cell surface in association with a self allele product which fits the receptor on the responding T cell.

The relationship between type I diabetes and specific HLA region alleles is complex. There is a strong positive relationship with HLA-DR3 and DR4 and a strong negative relationship with DR2 alleles. Individuals with either or both of these antigens have a greater chance of developing diabetes than do individuals who lack the antigen(s).

Indeed, 90% of whites with type I diabetes have one or both of these antigens. However 40% of patients without diabetes also possess one or both antigens. There is an even stronger relationship to type I diabetes when certain DQ alleles are considered together with DR loci. Some DQ alleles including HLA-DQA1*0201 and HLA-DQB1*0602 appear to provide protection from type I diabetes even in the presence of DQ susceptibility alleles.

1.4.1.2. Environmental Factors

The role of environmental factors in human type I diabetes is uncertain. As noted earlier, the fact that a significant proportion of monozygotic twins remain discordant for diabetes (one twin with, the other without) has suggested that nongenetic factors are required for expression of diabetes in humans. However, the potential genetic diversity between such identical twins apparently eliminates the need to include environmental events in the disease sequence. Yet, the prevailing view is that environmental factors at least influence the probability of type I diabetes developing in a given person.

One potential environmental influence is neonatal and early infant nutrition. There is a reciprocal relationship between breast-feeding and frequency of type I diabetes. It has been proposed that consumption of cow milk proteins, particularly early in life, may lead to initiation of the immunologic attack against pancreatic islet β cells and increase susceptibility to type I diabetes. Alternatively, consumption of breast milk may lead to disease protection (Skyler, 1997).

Several chemical toxins have a potential for destroying β cells, including nitrosourea compounds (eg, streptozotocin). One study suggested that maternal

consumption of nitrates and nitrites around the time of conception may influence eventual development of type I diabetes. The developing embryo, when exposed to toxins, may suffer an initial β-cell insult, enhancing the risk for diabetes which appears years later.

Exposure to a variety of viruses may influence development of type I diabetes. A number of human viruses can infect and damage β cells *in vitro*. The role of viruses in disease pathogenesis is unclear. However, type I diabetes develops in 15% of children with congenital rubella. Presumably viral infections of the pancreas could induce diabetes by two mechanisms: direct inflammatory disruption of islet cells and/or induction of an immune response. Environmental factors may influence the immune response through molecular mimicry. There is homology between glutamic acid decarboxylase and Coxacki protein P2-C; insulin and a retrovirus sequence; a 52-kd islet protein and a rubella protein; and the β-cell surface protein ICA-69 and a 17-amino-acid sequence of bovine serum albumin.

1.4.1.3. Immunologic Factors

Specific cell mediated T-lymphocyte immune processes appear responsible for the destruction of β cells, although the exact mechanisms have not yet been clearly defined. One theory is based on the appearance of class II molecules on the surface of the insulin-producing β cells. The idea is that these cells do not normally express D-region products but that in response to a virus (probably in the presence of IFN-γ and TNF-β), expression is induced. This would presumably allow the cell to function in antigen presentation utilizing either self or foreign antigen. Thus, immune activation seems to involve presentation of a diabetogenic peptide to the immune system, and activation of a T-helper

1 (Th1) subset of $CD4^+$ T lymphocytes. The cytokines produced by Th1-cell activation, IL-2 and IFN-γ, activate cytotoxic T lymphocytes and macrophages to kill β cells. The killing mechanism involves oxygen free radicals, nitric oxide, destructive cytokines (IL-1, TNF-α and β and IFN-γ), and $CD8^+$ cytotoxic T lymphocytes that interact with a β-cell autoantigen-MHC class I complex. Once initial destruction commences, secondary and tertiary immune responses are activated, compounding interpretation of the sequence of events.

The cell-mediated immune reaction is seen as a mononuclear cell infiltration of islets, insulitis, and is a pathogenomonic lesion seen in pancreases examined near the time of onset of type I diabetes. However, at the time of diagnosis of type I diabetes, only a few islets show the insulitis lesion. Most islets are "pseudoatrophic," small islets without mononuclear infiltration and devoid of β cells, but with intact glucagon-secreting α cells and somatostatin-secreting δ cells. Presumably these islets have already had their β cells destroyed.

A number of circulating antibodies are detected at diagnosis of type I diabetes, including cytoplasmic islet cell antibodies, insulin antibodies, antibodies directed against the enzyme glutamic acid decarboxylase and a variety of others. These antibodies usually are not thought to mediate β cell destruction by humoral mechanisms. Rather, it is likely that as β cells are destroyed, multiple antigens are exposed to the immune system, with generation of antibodies directed against these components. Thus, these antibodies serve as markers of immune activity or of β-cell damage, and are useful in heralding the disease process by the appearance of various antibodies, including cytoplasmic islet cell

antibodies, insulin antibodies, anti-glutamic acid decarboxylase antibodies, several years before the overt clinical onset of hyperglycemia and type I diabetes. As β-cell function is lost and diabetes evolves, antibodies tend to decrease in titer or disappear.

1.4.2. Type II, Non-Insulin Dependent Diabetes

Little progress has been made in understanding the pathogenesis of non-insulin dependent diabetes mellitus. Although the disease runs in families, modes of inheritance are not known. An HLA relationship has not been identified in type II NIDDM, and autoimmune mechanisms are not believed to be operative. However, the genetic influence is powerful, since the concordance rate for diabetes in monozygotic twins with type II disease approaches 100%. The risk to offspring and siblings of patients with NIDDM is higher than type I diabetes (5-10%). Nearly 40% of siblings and 33% of offspring eventually develop abnormal glucose tolerance.

There are two major pathogenic mechanisms operative in type II diabetes: impaired islet β-cell function (impaired insulin secretion), and impaired insulin action (insulin resistance or decreased insulin sensitivity). In most patients both defects are present, however, different patients manifest variable degrees of impairment in insulin secretion and insulin action. The primacy of the secretory defect *vs* insulin resistance is not established. However, current thinking is that there are separate genetic defects responsible for each of these. There is a progressive decrease in insulin sensitivity with age and insulin resistance is induced by adiposity and by a sedentary lifestyle. Thus it is not surprising that type II diabetes usually begins after 40 years of age and has an increased prevalence in older people, particularly those who are overweight or sedentary.

Moreover, hyperglycemia and elevated free fatty acids can further impair both β-cell function and insulin action, thus creating a vicious cycle that aggravates hyperglycemia.

1.4.2.1. Impaired Islet β-cell Function

The impairment in β-cell function is manifest in at least three ways. ***First***, there is blunted or absent first-phase insulin response to glucose, so that insulin secretion is delayed and fails to restore prandial glycemic excursions in a timely manner. The first phase of the normally biphasic insulin response is the critical determinant of the magnitude of hyperglycemia after carbohydrate intake. The decrease in first-phase insulin secretion results in an overall delayed insulin secretory response to glucose. Although variable, in most circumstances second-phase insulin response is sufficient to restore postprandial plasma glucose excursions to basal (preprandial) levels before the next meal, albeit after a prolonged interval. ***Second***, there is a decreased sensitivity of insulin response to glucose such that hyperglycemia may fail to trigger an appropriate insulin response. The insulin response to glucose is attenuated so that the β cell shows a relative blindness to hyperglycemia, failing to generate an adequate insulin response. ***Third***, there is decreased overall insulin secretory capacity, particularly in more severe type II diabetes.

The impairment in insulin secretory response is not static, but dynamic, such that chronic hyperglycemia may itself aggravate the impairment in insulin secretion so called "glucose toxicity." Thus, with progressive hyperglycemia in type II diabetes, there is concomitant deterioration in insulin secretory response. Moreover, and most important, when there is correction of hyperglycemia, there is some reversal of the impairment in endogenous insulin response to a meal challenge (i.e. demonstrable improvement in insulin

secretion). Attainment of glucose control, therefore, facilitates maintenance of glucose control. The mechanism of glucose-induced β-cell dysfunction is not known, but chronic exposure to high glucose has been shown to impair K^+-channel function and to reduce expression of the β-cell glucose transporter, GLUT2. Amylin, a peptide, which is cosecreted with insulin, might play a potential role in the pathogenesis of type II diabetes. In normal islets amylin is copackaged with insulin in β cell granules. However, in patients with NIDDM, amylin is deposited outside the β cells. A secretory defect of amylin and its local accumulation in the islets of type II diabetics might be a cause of insulin secretory defects in type II diabetes.

1.4.2.2. Impaired Insulin Action

Patients with type II diabetes also have impaired insulin action (insulin resistance) at target cells. Insulin resistance may be defined as being present whenever normal concentrations of insulin elicit a less-than-normal biologic response. Quantitatively, the impairment of insulin action principally affects skeletal muscle, specifically the non-oxidative pathway of glucose metabolism, resulting in diminished glucose storage (glycogen synthesis). To a lesser degree there is also reduced glucose oxidation. There is also apparent hepatic resistance to insulin action, with enhanced hepatic glucose production not inhibited by insulin.

In patients with type II diabetes, defects have been identified in insulin receptor binding of insulin, in receptor tyrosine kinase activity for both autophosphorylation and phosphorylation of substrate, in translocation of GLUT4 glucose transporter from the intracellular pool to the plasma membrane and activation therein, and in the total number

of glucose transporters per cell. However most of these defects are reversible with one or another intervention. The primary cellular mechanisms responsible for insulin resistance in type II diabetes are still being clarified.

It has also been recently demonstrated that amylin co-secreted with insulin is detectable in the peripheral circulation, and that pharmacological amounts of amylin were able to induce peripheral insulin resistance in rats *in vivo*. However, the functional significance of peripheral amylin concentrations in man remains to be determined.

A rare form of clinically mild type II NIDDM is due to production of an abnormal insulin that does not bind well to insulin receptors. Such persons respond normally to exogenous insulin.

1.4.2.3. Increased Insulin Clearance

In addition to reduction in insulin secretion from islet β cells, the lower plasma insulin levels observed in subjects with NIDDM could also be due to increased insulin clearance. This is supported by the fact that patients with NIDDM had lower steady-state plasma insulin concentrations achieved during glucose clamp studies. It has been shown that some subjects with insulin resistance, including Pima Indian patients with NIDDM, exhibit an accelerated rate of intracellular insulin degradation (Vasquez *et al.*, 1985). This has previously been proposed as a possible cause of the so-called post-receptor mechanism of insulin resistance. Furthermore, increased rates of insulin degradation have been found in several patients.

1.5. Pharmacokinetics in Diabetes Mellitus

The influence of diabetes mellitus on drug disposition has received relatively little attention. Complications of the disease include an increased incidence of ischaemic heart disease, hypertension, renal disease and infections often requiring specific drug treatment. The pathophysiological changes and alterations in glucose homeostasis associated with diabetes mellitus may have fundamental effects on basic cellular processes, resulting in altered handling of many drugs.

1.5.1. Absorption

Disorders of the gastrointestinal tract, such as diarrhea, constipation and delayed gastric emptying due to gastroparesis, may alter the rate and extent of absorption of drugs given orally. However, the extent of the influence of the disease on drug absorption may depend on the severity, duration and type of disease. The absorption of drugs having localized carrier-mediated transport systems may be highly sensitive to conditions such as gastroparesis. The extent of absorption of metoclopramide administered orally to diabetic patients with gastroparesis was found to be similar to healthy subjects (O'Connell *et al.*, 1987). Glipizide was found to be completely absorbed when administered to patients with NIDDM (Wahlin *et al.*, 1982). The absorption of tolazamide was 26% slower in patients with asymptomatic autonomic neuropathy than in healthy subjects (Della-Colleta *et al.*, 1988). The extent of absorption of ampicillin was found to be decreased only in patients with poorly controlled IDDM, but not in NIDDM or patients with well controlled diabetes (Adithan, 1989).

1.5.2. Distribution

This area has not been systematically studied, but differences would be anticipated as a consequence of the known alterations in protein binding. Diabetes can potentially influence drug binding in three ways: by changing the amount and concentrations of circulating proteins such as albumin and α_1-acid glycoprotein (Miller *et al.*, 1990; O'Connor, 1987); by increasing the blood concentration of substances which inhibit drug binding; and by changing the conformation or structure of plasma proteins, thereby reducing drug binding affinity. However, the extent of binding to plasma proteins is influenced by the degree of control of the disease, the duration of diabetes and the presence of detected or undetected complications of disease such as renal or hepatic failure. Blood concentrations of lipoproteins and proteins are known to be different in diabetic and control rats (Miller *et al.*, 1990; Kostner and Karadi, 1988).. It is shown that 6-week untreated STZ-induced diabetes in rat resulted in significantly increased (three fold) plasma levels of α_1-acid glycoproteins with no change in plasma albumin levels (Miller *et al.*, 1990). In diabetic patients, however, incresed free fraction of acidic drugs was partially attributedit to decreased plasma albumin levels (O'Connor, 1987). It is also well known that free fatty acids are elevated in the plasma of patients with either IDDM or NIDDM (Fraze *et al.*, 1985). Elevated free fatty acids, and glycosylation of plasma protein in diabetes mellitus may affect the extent of binding of basic and acidic drugs (Gwilt *et al.*, 1991). In addition, extra- and intracellular volumes are elevated in experimental diabetes mellitus (Harvey *et al.*, 1988). Hence, the volume of distribution of drug may be altered in diabetic states. The binding of valproic acid was found to be

significantly decreased in patients with IDDM (Grainger-Rousseau *et al.*, 1989). A linear relationship was also observed between the free fraction of valproic acid and serum FFA concentration (Gatti *et al.*, 1987). Similar findings have been reported for diazepam (McNamara *et al.*, 1988). It seems that drugs known to compete with FFA for albumin binding sites will have lower binding in diabetes. It has been demonstrated that phenytoin binding in insulin-dependent diabetic children was diminished possibly due to albumin glycation (Kemp *et al.*, 1987).

1.5.3. Metabolism

There have been numerous studies on the effect of diabetes on hepatic drug biotransformation in humans and animals. The direction and extent of the effect, however, are dependent on several factors such as the acuteness and type of the disease, duration of diabetes, gender, species and the substrate under study. Elimination of antipyrine, a marker for hepatic intrinsic clearance, was found to be faster in patients with treated IDDM (Daintith *et al.*, 1976; Zysset *et al.*, 1988) and also in non-insulin dependent diabetic patients with normal liver function (Salmela *et al.*, 1980). On the other hand, the clearance of this drug in patients with NIDDM was significantly reduced (Zysset *et al.*, 1988, Pirttiaho, 1984). A similar effect was found in streptozotocin (STZ)-induced diabetic rats (Mahachai *et al.*, 1988). The metabolism of aminopyrine was shown to be slower in both alloxan- and STZ-induced diabetic male rats (Toda *et al.*, 1987) and faster in female rats (Kato *et al.*, 1970). Paracetamol metabolism was unchanged in IDDM but decreased in NIDDM (Adithan, 1988[a]). The metabolism of theophylline, however, was increased in IDDM and unchanged in NIDDM (Adithan, 1988[b], 1989).

1.5.4. Renal Excretion

Kidney glomerular hyperfiltration has been documented in diabetic patients during the first decade of the disease (Bell, 1991; Mogensen *et al.* 1971,1983). This observation has also been demonstrated in animal models of IDDM (Jenson et al. 1981; Carney *et al.*, 1979; Craven *et al.*, 1987). The glomerular filtration rate (GFR), however, significantly decreases in diabetic patients with a longer duration of disease (Mogensen *et al.*, 1971). The elevation of GFR persists during the first decade despite insulin therapy and adequate metabolic control. This may possibly be related to the elevated capillary wall area volume and/or glomerular cell volume seen in diabetics (Osterby and Gundersen, 1975).

Consistent with these findings, renal clearance of some drugs may be changed in insulin treated diabetics. For instance, a marked increase in clearance of the renally excreted drugs penicillin (Madacsy *et al.*, 1975) and carbenicillin (Madacsy *et al.*, 1976) has been reported in diabetic patients under treatment with insulin. It should be noted that any study design to compare the renal clearance of drugs in diabetes must allow for different stages of diabetic renal disease. It would be inaccurate to draw conclusions about the effects of diabetes on renal clearance without differentiating between short and long term diabetes.

Change in renal clearance (Cl_r) can also occur apart from inherent changes in renal function. For example, highly bound drugs may demonstrate an increase in Cl_r due to decreased protein binding. Drugs with urine-flow dependent Cl_r, may demonstrate increased Cl_r due to the polyuria present in early diabetes.

2. INFLAMMATION

2.1. Background

Inflammation is the characteristic (non-specific protective) response of the body to tissue injury from any cause. Detailed descriptions of inflammation are found in relevant text books such as the Textbook of Rheumatology (Kelly *et al.*, 1981) and Anderson's Pathology (Mari, 1990) in which the following information was obtained.

Whenever tissue is injured, a series of events referred to as the inflammatory response are activated in an attempt to limit tissue damage by noxious agents. Cellular injury may be caused by microorganisms (bacteria, viruses and yeasts), trauma (mechanical forces), extreme temperatures, chemical agents, microbial products (endotoxin, exotoxin) and other inflammatory agents.

According to its duration, inflammation is classified as acute or chronic. Inflammation is referred to as acute when it lasts for days or less than a few weeks. Some examples include viral and bacterial infections such as typhoid fever and diphtheria, and hypersensitivity reactions. The persistent presence of such infections, hypersensitivity, or noxious agents, will often result in a chronic condition even after the initial acute process subsides. Inflammation is classified as chronic when its duration is prolonged (i.e. months, years). Specific examples of chronic inflammatory conditions include rheumatoid arthritis, syphilis, Reiter's syndrome and Crohn's disease.

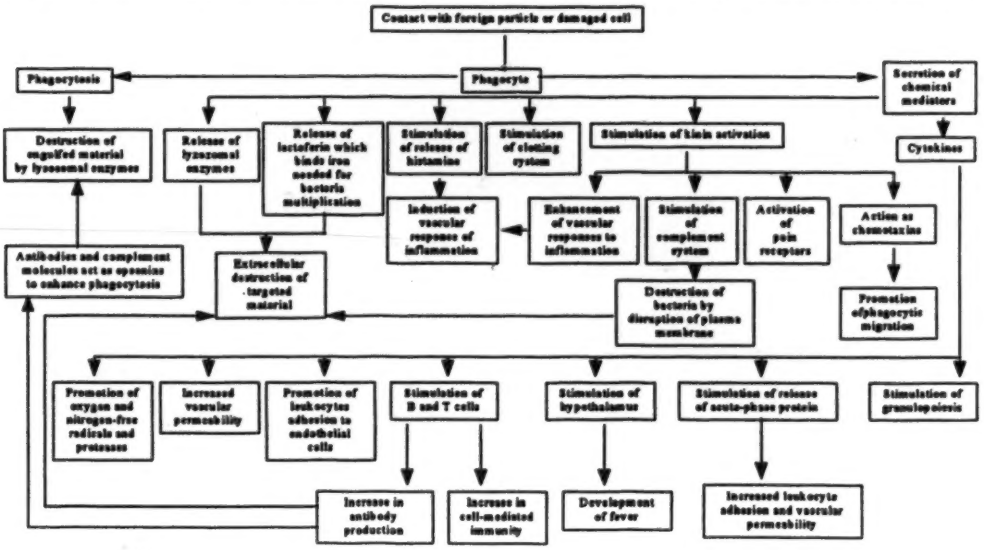
The main events comprising the acute inflammatory response include vasodilatation, exudation of plasma, and migration of neutrophils to the injured tissue. In chronic conditions there is a subsequent accumulation of macrophages, lymphocytes and

plasma cells at the site of inflammation which is accompanied by necrosis and proliferation of tissue cells. Infiltration of macrophages to the site of inflammation is also apparent. However, although this is transient in acute conditions, a persistent proliferation of macrophages is seen in chronic conditions. Activated macrophages/monocytes release many chemicals including the hormone-like proteins called cytokines which function as mediators of the inflammatory response. Action of the inflammatory cytokines (IL-1, IL-6 and TNFα) may be local (paracrine) and/or systemic (endocrine). Paracrine effects of inflammatory cytokines include promotion of adherence of leukocytes to endothelial cells, increased vascular permeability, and stimulation of neutrophils and macrophage/monocytes (phagocytes) to produce nitrogen and oxygen free radicals and proteases which kill bacteria. The endocrine (systemic) response produced by IL-1, IL-6 and TNFα includes both pro- and anti-inflammatory actions. Proinflammatory actions of IL-1, IL-6 and TNFα are exerted on the hypothalamus to produce fever and potentiate chemotaxis, phagocytosis, and free radical production, and also on muscle and liver to produce hepatic acute phase proteins that increase both leukocyte adhesion to endothelial cells and vascular permeability. Anti-inflammatory actions of IL-1, IL-6 and TNFα are exerted on muscle and liver to produce other hepatic acute phase proteins that inhibit proteases, and on the hypothalamic-hypophyseal-adrenal axis to produce corticosteroids that inhibit T-and B-lymphocyte functions, chemotaxis, phagocytosis, and free radical production by phagocytes. Some of the acute phase proteins include fibrinogen, serum amyloid A, C-reactive protein, α_1-antichymotrypsin, α_1-antitrypsin, α_1-acid glycoprotein, haptoprotein and the complement components. The acute phase proteins play a role in

inflammation and healing. Furthermore, changes in the serum levels of acute phase proteins may be used to assess disease activity and therapeutic intervention.

Animal models of inflammation have been extensively utilized by pharmacologists to study drug therapy. An acute inflammatory response is often elicited by subcutaneous injection of chemicals such as turpentine into the rat or rabbit (Whitehouse, 1988). Inflammation activity and successful therapeutic intervention is evaluated by measurement of physical indices such as paw swelling or by determining concentrations of acute phase proteins. However, difficulties arise when information obtained in these studies are extrapolated to intervention in disease associated with chronic inflammation. Beck and Whitehouse (1974) found a differential effect of acute and chronic inflammation on drug disposition. Consequently, the rat adjuvant arthritis animal model is commonly employed. Injection of an adjuvant prepared from several species of mycobacteria induces a chronic inflammatory disease resembling rheumatoid arthritis (Whitehouse, 1988).

Figure 1-1. Biological Roles of Phagocytes (Monocytes, Neutrophils and Macrophages).


Contact with foreign particle or damaged cell
Phagocytosis
Phagocyte
Secretion of chemical mediators
Destruction of engulfed material by lysosomal enzymes
Release of lysozomal enzymes
Release of lactoferin which binds iron needed for bacteria multiplication
Stimulation of release of histamine
Stimulation of clotting system
Stimulation of kinin activation
Cytokines
Induction of vascular response of inflammation
Enhancement of vascular responses to inflammation
Stimulation of complement system
Activation of pain receptors
Action as chemotaxins
Antibodies and complement molecules act as opsonins to enhance phagocytosis
Extracellular destruction of targeted material
Destruction of bacteria by disruption of plasma membrane
Promotion of phagocytic migration
Promotion of oxygen and nitrogen-free radicals and proteases
Increased vascular permeability
Promotion of leukocytes adhesion to endothelial cells
Stimulation of B and T cells
Stimulation of hypothalamus
Stimulation of release of acute-phase protein
Stimulation of granulopoiesis
Increase in antibody production
Increase in cell-mediated immunity
Development of fever
Increased leukocyte adhesion and vascular permeability

2.2. Drug Disposition in Inflammatory States

Possible causes of inflammation-induced increases in drug concentrations include increased absorption, altered distribution and/or a reduction in elimination.

2.2.1. Absorption

It is thought that changes in intestinal mucosal integrity due to the disease or drug therapy may influence the absorption of xenobiotics (Mielants *et al.*, 1991). Altered absorption, however, has not been observed to be a major factor in inflammation-induced changes in drug disposition. One report (Kirch *et al.*, 1982) noted a decrease in the absorption of atenolol in patients suffering from an acute respiratory infection. Nevertheless, the proposed change in atenolol absorption requires confirmation, since total urinary recoveries of atenolol were unchanged; this is an important observation as this drug is primarily eliminated via the renal route. Oral absorption of propranolol was found to be the same in treated adjuvant arthritic rats as compared to healthy animals (Key *et al.*, 1986). Altered absorption in inflammation has not been reported for other drugs.

2.2.2. Distribution

Inflammatory conditions are associated with altered plasma protein concentrations. Hypoalbuminemia, which is often observed in arthritic subjects, may affect protein binding of acidic drugs. In patients with rheumatoid arthritis, the pharmacokinetics of the highly protein bound NSAID, naproxen, were found to be significantly altered due to an arthritis-induced reduction in plasma albumin concentration (Van Den Ouweland *et al.*, 1987). Van Den Ouweland observed significantly increased free fractions of naproxen in arthritic patients which resulted in both a 60% increase in the volume of distribution and a 40%

increase in the apparent clearance of naproxen. Plasma concentrations of the acute phase protein, α_1-acid glycoprotein are also greatly increased in inflammatory conditions. Basic drugs are mainly bound to this protein, and therefore, protein binding of these agents are often increased in disease such as arthritis. Laethem (1994) demonstrated a decrease in the unbound fraction from 0.068 to 0.02 for R-propranolol and 0.26 to 0.14 for S-propranolol in control *vs* endotoxin treated rats.

2.2.3. Metabolism

The impairment of drug metabolism in experimental models of inflammation has been well established. Following induction of adjuvant arthritis in rats, liver microsomal oxidative enzyme activity and cytochrome P-450 content were reduced (Sakai *et al.*, 1992; Ferrari *et al.*, 1993; Moreno *et al.*, 1987). *In vivo* studies also demonstrated decreased paracetamol glucuronidation and increased phenobarbitone toxicity in arthritic rats, resulting from an impaired liver metabolism. In other experimental models of inflammation significant reduction in hepatic enzyme activity and cytochrome P-450 content resulting in altered metabolic clearance of hexobarbital and tolbutamide were also found (Ishikawa *et al.*, 1991, Parent *et al.*, 1992). Propranolol levels have been reported to be significantly higher than normal in patients with inflammatory diseases (Schneider et al., 1979, 1981) as well as in rats afflicted with inflammation (Belpaire *et al.*, 1989; Piquette-Miller and Jamali, 1993, 1995). These increases have been explained by both decreased metabolism and increased protein binding.

More recently, the role of individual inflammatory mediators in altered drug disposition has been addressed. Reports indicate a depressive effect of interleukin 1 (IL-

1), , TNF-α, and IL-1β and IL-2 on drug metabolizing enzymes (Ferrari *et al.*, 1993; Dinerallo, 1992; Ansher *et al.*, 1992). Furthermore, incubation of hepatocytes with IL-1 has been found to decrease cytochrome P-450 in a dose-dependent manner (Sujita *et al.*, 1990). Recently, the influence of i.v. administration of the inflammatory mediator, IL-1β on the pharmacokinetics of propranolol was investigated in the rat (Vermeulen *et al.*, 1993).

2.2.4. Renal Excretion

Abnormalities in renal function are frequently noted due to disease and/or therapy induced changes in rheumatoid arthritis (Mody *et al.*, 1987). Furthermore, administration of NSAIDs can reduce renal function through inhibition of renal prostaglandin synthesis (Downie, 1991). However, few studies have examined the effect of inflammation on the renal clearance of drugs. Inflammatory bowel disease was not found to influence the plasma concentration profiles of the renally excreted β-blocker practolol (Parsons *et al.*, 1976).

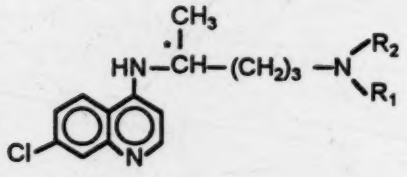
3.HYDROXYCHLOROQUINE

3.1. Chemistry of Hydroxychloroquine

The compound (±)-7-chloro-4-(5-(N-ethyl-N-2-hydroxyethylamino)-2-pentyl) aminoquinoline, $C_{18}H_{28}ClN_3O$, hereafter known as HCQ is a slightly yellow powder with a molecular weight of 335.87 and melting point of 89-91 °C. Hydroxychloroquine sulfate (Plaquenil, Sanofi Winthrop Pharmaceuticals, New York, NY), $C_{18}H_{26}ClN_3O_5S$, is a white crystalline powder and is odourless with a bitter taste. Hydroxychloroquine sulfate is freely soluble in water and practically insoluble in alcohol, chloroform, and ether (Merck

Index, 1989). The chemical structure of HCQ is given in figure 1-2. Hydroxychloroquine possesses a chiral center and is used clinically as the racemate. Currently, little is known about the relative anti-arthritic activities of the HCQ enantiomers, although recent advances have permitted the quantitative analysis of the enantiomers in biological fluids.

Figure 1-2. Structures of chloroquine, HCQ, and their major metabolites. Chiral centre is denoted by the asterisk. ([a] HCQ metabolites, [a,b] HCQ and chloroquine metabolites).



Compound	R_1	R_2
Hydroxychloroquine	CH_2CH_3	CH_2CH_2OH
Chloroquine	CH_2CH_3	CH_2CH_3
Desethylchloroquine[a,b]	CH_2CH_3	H
Desethylhydroxychloroquine[a]	H	CH_2CH_2OH
Bisdesethylchloroquine[a,b]	H	H

3.2. Clinical Usage

3.2.1. Rheumatoid Arthritis

The beneficial effect of 4-aminoquinoline compounds in the management of connective tissue disorders was discovered by serendipity (Mackenzie, 1983; Dubios, 1978). Subsequent controlled studies have consistently demonstrated the effectiveness of HCQ in the treatment of various connective tissue disorders (Hamilton & Scott, 1962;

Mainland and Sutcliffe, 1957; The HERA study group, 1995; Clark *et al.*, 1993). Early controlled trials showed suppression of joint inflammation (i.e. tender and swollen joint count) and morning stiffness in rheumatoid arthritis patients treated with HCQ (Hamilton & Scott, 1962; Mainland and Sutcliffe, 1957). However, in these studies higher doses of HCQ were used than those currently recommended for maintenance therapy. Recent multicentre, well-controlled, double-blind studies in patients with early rheumatoid arthritis have also demonstrated a decrease in joint inflammation and stiffness with a low level of side effects (the HERA study group, 1995; Clark *et al.*, 1993). In these studies, the effect of HCQ on the incidence of new joint erosion or progression of joint erosion was not assessed. Hydroxychloroquine was also shown to be effective in decreasing joint inflammation in children with juvenile rheumatoid arthritis (Laaksonen *et al.*, 1974). It is found that a lower risk of toxicity is associated with HCQ treatment than with commonly employed disease-modifying antirheumatic drugs including intramuscular gold, D-penicillamine, azathioprine, or methotrexate (Fries *et al.*, 1993).

3.2.2. Systemic Lupus Erythematosus and Sjögren's Syndrome

A controlled study of 25 systemic lupus erythematosus (SLE) patients receiving HCQ (400 mg/day) indicated complete clearing or marked improvement in skin rash in 68% of the patients (Ruzicka et al., 1992). Additional controlled studies of HCQ in patients with SLE have demonstrated significant improvement in arthritis, myalgias, pleuritic symptoms, and joint pain (Dubios, 1978; Rudnicki et al., 1975). Also, HCQ may permit the use of lower doses of systemic corticosteroids in patients with SLE (Dubios, 1978).

One controlled study of HCQ reported improvement in patients with primary Sjögren's syndrome (Fox et al., 1988). These patients were selected for entry into the study on the basis of active lupus-like signs (e.g., active arthritis, rash), recurrent swelling of parotid glands/lymph nodes, and elevated acute phase reactants (i.e., ESR, IgG). These extraglandular manifestations in patients with Sjögren's syndrome were significantly improved by use of HCQ (7 mg/kg/day).

3.2.3. Other Uses of Hydroxychloroquine

Improvement of serum cholesterol and liver-associated enzymes in patients treated with HCQ have been reported (Wallace, 1994). These include a decrease in serum levels of cholesterol by approximately 10% and an increase in low-density lipoprotein receptors (Wallace *et al.*, 1987; Lorber *et al.* 1985; Benyen, 1986). This effect may be important in the prevention of atherosclerotic complications in patients with SLE treated with corticosteroids or with renal disease.

An interesting antithrombotic effect was reported in a study of over 5000 patients receiving total hip replacement (Carter *et al.*, 1971; Johnson *et al.*, 1979). Hydroxychloroquine may block platelet aggregation and adhesion without prolongation of bleeding time and wound healing (Loudon, 1988). A dose of HCQ of 600 mg/day was used for 1 to 2 weeks postoperatively and found to have efficacy comparable to heparin in prevention of thrombosis (Wallace, 1987). This antithrombotic effect may be beneficial in SLE patients since a retrospective study demonstrated a significantly lower frequency of thrombotic events in patients receiving antimalarial medications (Wallace et al. 1990).

Hydroxychloroquine has been used in a wide variety of dermatologic conditions. Of particular importance is the use of this medication in relatively high doses in discoid lupus (Dubios, 1987).

The study of HCQ (6-7 mg/kg/day) in the treatment of corticosteroid-dependent asthma was reported to allow a 50% decrease in steroid usage and a decrease in serum IgE levels (Charous, 1990). Hydroxychloroquine has also been used to treat patients with refractory noninsulin-dependent diabetics and its use led to improved control of hyperglycemia (Quatraro *et al.* 1990). In addition, *in vitro* and *in vivo* studies have demonstrated that HCQ has the ability to inhibit replication of various strains of HIV-1 and may be a potentially useful drug either as a sole agent or as adjunctive therapy in the treatment of HIV-1 infection (Sperber *et al.* 1993[a]; Ornsstein and Sperber, 1996). Hydroxychloroquine is usually considered as second line therapy for patients with rheumatoid arthritis, after the non-steroidal anti-inflammatory drugs. However, this drug is an attractive alternative for use in the early stages of RA or in combination with other anti-arthritic agents for more severe disease. Current trends are towards use of the drug earlier, after diagnosis, in an attempt to minimize damage from inflammation (Wilske, 1989; the HERA study group, 1995).

3.3. Mechanism of Action

3.3.1. In Malaria

At least three mechanisms have been proposed for the antiparasitic effects of antimalarial compounds. ***First***, antimalarial compounds may elevate the intracellular pH of vesicles within the parasite and interfere with the enzymatic breakdown of hemoglobin

(a food source for the parasite) and the assembly of malaria-specific proteins (Homewood *et al.*, 1972). ***Second***, the heme metabolite, ferriprotoporphyrin IX is made when the malarial parasite enters the erythrocytic phase (schizont) of replication. Antimalarial compounds bind with strong affinity to ferriprotoporphyrin IX and the complex is toxic to the schizont phase of the malarial organism (Chou *et al.*, 1980). ***Finally*** a recent report describes a malaria encoded enzyme that polymerizes heme molecules (and prevents their toxic effect on the infected erythrocyte). Chloroquine interferes with this malaria-induced enzyme (Slater and Cerami, 1992).

3.3.2. In Rheumatoid Arthritis

Hydroxychloroquine has been used for many years for the treatment of rheumatic diseases, yet its mechanism of action in these disorders remains controversial. Hydroxychloroquine is extensively concentrated intracellularly, particularly in acidic cytoplasmic vesicles throughout the body (Tett *et al.*, 1990, 1994, Brocks *et al.*, 1994, French *et al.*, 1992). The various functions of this vesicular system, such as posttranslation modification of proteins (glycosylation in the Golgi apparatus), digestion of membrane lipids, protein degradation, turnover of cell surface receptors, and assembly of macromolecules in the endosomes may be changed as a result of the reduced pH of vacuole and/or by effects on acid proteases and glycosidases function and release. This has particularly important implications for monocytes and the macrophage cells that require precise pH to digest foreign proteins in their lysosomes and assembling them into multichain complexes in their endosomes. The important antirheumatic effects result from drug accumulation within the cells of the immune system. The therapeutic benefits of

HCQ in arthritis are believed to result from its interference with "antigen processing" in macrophages and other antigen-presenting cells. As a result HCQ diminishes the formation of peptide-MHC protein complexes required to stimulate $CD4^+$ T cells resulting in down-regulation of the immune response against autoantigenic peptides (Mackenzie, 1983; Fox and Kang, 1993). Studies have shown that the aminoquinoline compounds inhibit T lymphocyte responsiveness to mitogens in peripheral blood mononuclear cells obtained from patients with rheumatoid arthritis (Hurvitz and Hirchorn, 1993). In addition, inhibition of chemotaxis (Hurst *et al.*, 1988), reduced production of reactive oxygen species by phagocytic cells (Hurst *et al.*, 1988), and diminished cytokine release (Sperber *et al.*, 1993[b]) from monocytes/macrophages have been proposed as contributory mechanisms of action. Recent studies with purified phospholipase A2 have shown that HCQ does not appear to inhibit phospholipase A2, an enzyme important in the generation of arachidonic acid (Matsuzawa and Hostetler, 1980). Studies examining the pharmacological mechanisms of action of HCQ in arthritis have mostly involved incubation of racemate with a suspension of white blood cells, followed by *in vitro* assessment of cell function (Harth, 1992).

3.4. Pharmacokinetics of Hydroxychloroquine

3.4.1. Absorption

Absorption of HCQ is incomplete with a mean absorption half-life of about 4 hours (Tett *et al.*, 1992). The mean fraction absorbed from the tablet was found to be similar (almost 70%) in fasting healthy volunteers (Tett *et al.*, 1989, 1992) and a group of patients with rheumatoid arthritis (McLachlan *et al.*, 1994). However, the interindividual

variability in fraction of dose absorbed was significant (range 25 to 100%) which may be a cause of therapeutic failure in some patients (Tett *et al.*, 1992,1993). The absorption half-life of 4 h is comparable to small intestine transit times, suggesting that the residence time in small intestine could be an important determinant of the extent of HCQ absorption (Tett *et al.*, 1989). An alternative explanation for incomplete HCQ bioavailability may be the first pass effect. A hepatic extraction ratio of 0.06 and 1 is obtained from the data of Tett *et al* (1988) for blood and plasma respectively. The small value of the extraction ratio estimated from blood data might be due to the fact that drug contained within blood cells would not be available for elimination. However, if the rate of cellular uptake of HCQ is slow, the drug would remain in the plasma and would be more available for extraction. On the basis of this explanation, the observed bioavailability (70%) may indicate that, following absorption, about 30% of HCQ remains in plasma by the time drug enters liver from the portal vein (Tett *et al.*, 1989). This interpretation is consistent with the finding that the *in vitro* uptake of HCQ into white cells is slow (equilibration time of about 1 h) (French *et al.*, 1987). The ratio of blood to plasma (B/P) tends to be lower at very early times following the infusion (up to 0.75 h), suggesting that overall distribution from plasma into cells is not rapid. Further work is needed to clarify this point. It is of potential clinical significance as this may be a source of variability in plasma concentrations between patients (Tett *et al.*, 1988).

3.4.2. Distribution

The major reason for the long elimination half-life of HCQ is primarily due to extensive tissue distribution, rather than to low clearance (667 mL/min based on plasma

data and 96 mL/min based on blood data, Tett *et al.*, 1988, 1989). Hydroxychloroquine is extensively taken up into tissues and is concentrated in the cellular fraction of whole blood (Tett *et al.*, 1990, 1994; Brocks *et al.*, 1994; French *et al.*, 1992). Evidence from animal studies indicates that accumulation in adipose tissue is not responsible for the large distribution of HCQ (McChesny, 1983).

In spite of many studies in both animals and humans, the mechanism of sequestration of HCQ into blood cells and tissues is not very clear,. It has been demonstrated that weak bases, such as HCQ, are accumulated in lysosomes through ion trapping mechanisms (MacIntyre & Cutler, 1988). These drugs are uncharged at the neutral pH of plasma and can easily diffuse across cell membranes (Krogstad *et al.*, 1987). Within the acidic vesicle of the cytoplasm of the cell, these drugs become protonated and are then unable to diffuse back across the cell membrane. This leads to increased drug concentration in most acidic cell vesicles (Homewood *et al.*, 1972). Since tissues with high accumulation ratios tend to be lysosome-rich, it is possible that *in vivo* accumulation is largely due to lysosomal content. For instance, homogenization of liver tissue (which also disrupts lysosomes) resulted in a large reduction of uptake to approximately the level of muscle (McIntyre and Cutler, 1988). Mathematical predictions based on cell and lysosomal volumes, and scale-up from known tissue distribution in animals, indicate that lysosomal trapping is not the only explanation for large volume of distribution (Tett *et al.*, 1990). For example, the drug accumulates in red cells by a combination of binding to intracellular materials, presumably hemoglobin, and ion-trapping due to the slightly acidic interior of the red cell relative to plasma. Accumulation, however, is much greater in

white cells and platelets. This appears to be due to the high lysosomal content of these cells (Tett *et al.*, 1990). Intracellular binding also occurs in pigmented tissues. Individual pigmented animals showed increased levels of drug in pigmented tissues, including melanocytes and retinal cells. Both HCQ enantiomers bind equally to the pigmented tissues of the eye in rabbits (Wainer *et al.*, 1994).

3.4.3. Plasma Protein Binding

Hydroxychloroquine is a basic drug and is only about 50% bound to plasma proteins, principally albumin (McLachlan *et al.*, 1993). The protein binding of this drug in the pooled plasma of healthy and arthritic subjects was found to be stereoselective in favour of the S enantiomer (Brocks *et al.*, 1994). The mean unbound fractions in healthy subjects were 66% and 47% and in arthritic patients were 56% and 41% for R and S enantiomers respectively (Brocks *et al.*, 1994). Reduced free fraction for both enantiomers of HCQ found in arthritic patients might be explained by significantly higher α_1-acid glycoprotein levels than in healthy subjects (Brocks *et al.*, 1994).

3.4.4. Blood and Plasma Concentration

Blood concentrations of HCQ are reported to be on average 7 to 8 times greater than plasma concentration due to extensive accumulation of the drug in white blood cells, platelets and erythrocytes (Miller *et al.*, 1991; Brocks *et al.*, 1994). The long half-life of HCQ can be attributed to extensive tissue uptake rather than to an intrinsic inability to clear the drug. Recently, Brocks *et al.* have shown that HCQ binds to platelets, reaching a concentration of approximately 50 times that in erythrocytes and 200 times that in leukocytes. However, the overall contribution of each fraction to the whole blood

concentration cannot be estimated from data generated in this study (Brocks *et al.*, 1994). The contribution of leukocytes, platelets, erythrocytes, and plasma to the total blood concentration for chloroquine, however, was reported to be 40, 35, 10 and 15% respectively (Bergqvist and Domeij-nyberg, 1983). Extensive accumulation of the drug in white blood cells, platelets and erythrocytes, technical difficulty in completely separatig platelets from plasma, and redistribution of drug from blood cells, may cause a large variability in the results using plasma as analyte medium (Tett *et al.*, 1988). Unknown amounts of platelets will remain in plasma with the centrifugation forces commonly used (250-2,000 g). However, 20,000 g for 30 min is needed to obtain platelet-free plasma (Malmgren and *et al.*, 1981). Whole blood appears to be the most reliable medium for quantification of HCQ concentrations. Whole blood is also preferred in animal studies which require low volume blood samples because it contains higher concentrations of HCQ than plasma. It is generally assumed that the pharmacological action of a drug is related to its unbound concentration in the blood. Thus, the unbound concentration would ideally be preferred for studies of the relationships between pharmacokinetics and drug effects. However, it is recommended that until a reliable separation procedure becomes available, pharmacokinetic data of HCQ be based on whole blood determinations (Tett *et al.*, 1988). The expected delay in the attainment of steady-state concentrations may be in part responsible for the slow therapeutic response observed with HCQ (Tett *et al.*, 1988).

3.4.5. Elimination

Hydroxychloroquine has a large total plasma clearance (833±379 mL/min), amounting to about 30% of plasma cardiac output (Tett *et al.*, 1988). Renal clearance

(211±97 mL/min) accounts for only 15 to 25% of total clearance (Miller *et al.*, 1991, Tett *et al.*, 1988). The hepatic clearance is similar in magnitude to the plasma flow rate to the liver and suggests an unusually efficient elimination process. The CL_r is substantially greater than the GFR indicating that renal processing of HCQ involves tubular secretion.

Hydroxychloroquine is metabolized primarily via the N-dealkylation pathway producing two major metabolites, desethylhydroxychloroquine and desethylchloroquine. These two metabolites are also dealkylated further to produce a primary amine, bis-desethylchloroquine. All three metabolites can be found in urine and plasma (Miller *et al.*, 1991). At steady-state, blood concentrations of desethylhydroxychloroquine reaches 50-65% and desethylchloroquine about 20% of parent drug concentrations (Miller *et al.*, 1991, Tett *et al.*, 1993). It remains unknown whether these metabolites play a role in antirheumatic activity.

3.4.6. Concentration-Effect Relationship

A relationship has not been demonstrated between plasma HCQ levels and efficacy in adults with rheumatoid arthritis taking HCQ for more than three months (Miller *et al.*, 1987). However, a positive relationship between blood HCQ concentrations and clinical outcomes was observed in arthritic patients (Tett *et al.*, 1993). In this study, patients with clinical measures indicating less active rheumatoid arthritis had statistically significant higher blood concentration of HCQ than those with more active disease (Tett *et al.*, 1993). The fact that it is difficult to separate cells from plasma completely and reproducibly may provide an explanation for this discrepancy. These data provide the first evidence of a concentration-response relationship for HCQ in rheumatoid arthritis for

individual disease activity measures. However, an unweighted summed score of disease activity did not correlate significantly with drug blood concentrations (Tett *et al.*, 1993).

3.4.7. Variability in Response

Significant interindividual variability in the fraction of oral dose of HCQ absorbed ranges from about 30 to 100 % (McLachlan *et al.*, 1993, Tett *et al.*, 1989, 1992). Also, after single intravenous doses, a two-fold range of clearance values has been calculated (Tett *et al.*,1988). Steady-state concentrations for a given dosage are proportional to the fraction of the dose absorbed and inversely proportional to clearance. Thus, a group of patients, (those exhibiting low bioavailability and /or high clearance of HCQ) may not respond to the therapy, simply because of subtherapeutic drug concentrations. Poor relationships have been reported between dosage and steady-state HCQ concentrations (Miller *et al.*, 1991, Tett *et al.*, 1993). Thus, pharmacokinetic variability is likely to contribute to the variability in clinical response because response appears to be related to the HCQ blood concentrations achieved.

3.4.8. Stereoselective Disposition

Hydroxychloroquine is a chiral agent which is clinically administered as a racemic mixture of equal quantities of the R and S enantiomers. Differential efficacy or toxicity of the two enantiomers of HCQ has not been reported. Three assays are currently available for separation of the enantiomers in biological fluids (Brocks *et al.*, 1992; Iredale and Wainer, 1992; McLachlan *et al.*, 1991).

Enantioselectivity in the pharmacokinetics of HCQ has been previously studied in eight patients with rheumatoid arthritis receiving chronic racemic HCQ treatment

(McLachlan *et al.*, 1993) and in healthy subjects following a single oral dose of the racemate (Ducharme *et al.*, 1995). It appears that S-HCQ is preferentially metabolized and excreted. Differences in absorption of the two enantiomers have not been found (McLachlan *et al.*, 1994). Higher steady-state concentrations of R-HCQ have been reported in blood and plasma, but the ratio of R to S enantiomers displays a two-fold variability between individuals (McLachlan *et al.*, 1993). Stereoselective protein binding has also been reported (Broks *et al.*, 1994; McLachlan *et al.*, 1993). Interindividual variability in stereoselective disposition could be another contributor to the observed variability in response if the enantiomers do have different therapeutic activities.

3.5. Side Effects

Ocular toxicity with HCQ was first reported in 1967 (Shearer and Dubios, 1967). In the time interval from 1960 to 1990, a total of 18 cases of retinopathy in patients receiving HCQ was reported either in the literature or to the Food and Drug Administration. In 16 of these cases, the dose of hydroxychloroquine was greater than 7 mg/kg/day (Bernstein, 1991). Thus, current recommendations for chronic antirheumatic use from the manufacturer indicate the use of HCQ at 6-7 mg/kg/day; the manufacturer has removed the indication of antirheumatic treatment for chloroquine due to risk of ocular toxicity (Salmeron and Lipsky, 1983). Corneal deposits, a reversible side effect, are relatively frequent in patients treated with chloroquine, but infrequent in HCQ-treated patients (Finbloom *et al.*, 1985). A retrospective review of 1500 patients using HCQ at a dose $\leq$ 7 mg/kg/day revealed no cases of retinopathy (Bernstein, 1991). Maculopapular rashes may occur in 3-5% of patients receiving antimalarial medications (Dubios , 1978).

Patients taking long-term HCQ may develop areas of hyperpigmentation in photo-exposed regions (Dubios , 1978).

4. HYPOTHESIS AND OBJECTIVES

4.1. Rationale for Hypotheses

Drug interactions are frequently characterized as being either pharmacokinetic or pharmacodynamic. Pharmacokinetic interactions influence the disposition of a drug in the body. The absorption, distribution, excretion or metabolism of a drug may be affected by the concomitant administration of a second drug, leading to differences in pharmacodynamics. Pharmacodynamic interactions are related to the pharmacologic activity of the interacting drugs. Mechanisms of pharmacodynamic interactions include synergism, altered cellular transport, and effects on receptor sites. An understanding of the mechanism involved with a particular interaction is essential in interpreting, preventing, treating and using specific interactions for therapeutic advantage. It is clear that either the therapeutic or toxic effects of a drug can be greatly modified by interactions with other drugs, foods, or endogenous substances. To determine the mechanisms involved in the observed pharmacodynamics, subsequent studies usually involve quantitative measurements on pharmacologic response. This can be easily quantitated through measures such as prothrombin times, serum glucose levels, blood pressure, or electrocardiograms.

Many types of interactions have been used to therapeutic advantage. There are many clinically significant examples of inhibition of drug metabolism in humans. Such inhibition leads to a reduction in the rate of metabolism and an increased elimination half-

life of the compound being affected. Inhibition of metabolism could be the therapeutic basis for some drugs.

Chloroquine and HCQ, which have long been used as antimalarial drugs, have more recently been used as disease-modifying agents in systemic and cutaneus lupus erythematosus and rheumatoid arthritis (Rynes, 1992; The HERA study group, 1995; Clark *et al.*, 1993). Numerous *in vitro* studies have used chloroquine as a tool to study the binding and the processes of insulin metabolism in target tissues. These studies have shown that chloroquine, which has the closest physicochemical similarity to HCQ (Fig. 1-2), reduces intracellular insulin degradation, increases intracellular insulin accumulation, slows receptor recycling and stimulates insulin-mediated glucose transport (Smith *et al.*, 1989; Cynober et al., 1987). These observations suggest that HCQ may behave in the same manner as chloroquine. A literature review on the effects of HCQ on glucose homeostasis reveals a small clinical trial in 38 patients with NIDDM resistant to standard therapy. A significant improvement in their glucose was demonstrated after six months of therapy with this drug (Quatraro *et al.*, 1988).

In general, HCQ is preferred over its analogue chloroquine, due to lower incidence of retinal damage (Finbloom *et al.*, 1985) and gastrointestinal toxicities (Tett *et al.*, 1990). Furthermore, the manufacturer has removed the indication of antirheumatic treatment for chloroquine due to the risk of ocular toxicity (Salmeron and Lipsky, 1983). Nevertheless, the physiological manifestations of HCQ in the non-diabetic state and potential hypoglycemic effect of this drug on insulin and glucose metabolism in animal models of diabetes needs to be determined. It seems essential to further study the mechanism of

HCQ action on glucose and insulin metabolism. In non-diabetic states the disturbance in glucose homeostasis induced by HCQ will probably be antagonized by a counter-regulatory increase in the concentrations of cortisol, glucagon, growth hormone, and catecholamines and/or increase in insulin secretion from β cells. Therefore, in non-diabetic subjects the normal homoeostatic mechanisms responsible for insulin-glucose regulation may compensate for possible HCQ-induced perturbation in insulin metabolism and prevent the onset of hypoglycemia. We were thus prompted to investigate the hypoglycemic action of HCQ in STZ-induced diabetic and healthy rats.

As the effect of HCQ on insulin and glucose metabolism is presumably a receptor and enzyme mediated process, this effect may be concentration dependent. Therefore, various doses of HCQ will be given to diabetic animals to further characterize the relationships between blood concentrations of HCQ and observed effects.

The uptake of insulin is mediated by specific insulin receptors in a number of tissues including liver. Once the insulin-receptor complex has been internalized, insulin undergoes rapid degradation (Smith *et al.* 1989). Insulin degradation, as reviewed by Duckworth and Kitabchi (Duckworth *et al.*, 1981, Duckworth, 1988), is accomplished through three enzymatic processes. Glutathione insulin transhydrogenase is a microsomal enzyme that catalyzes the degradation of insulin as well as other disulfide-containing proteins, by cleavage of disulfide bonds (Varandani *et al.*, 1972). This enzyme is not a major physiological degrading enzyme nor an obligatory initial step in insulin degradation (Duckworth, 1988). Lysosomal enzymes are involved in insulin degradation, however, the contribution of this system to insulin metabolism seems very small (Duckworth *et al.*,

1981). Insulin protease (insulinase), on the other hand, is a proteolytic enzyme which is specific for insulin (Brush *et al.* 1971; Duckworth 1988) and is predominantly (>90%) located in the soluble fraction of cell homogenates of rat tissues (Kitabchi *et al.*, 1972). Insulin protease accounts for more than 95% of all insulin degrading activity in human muscle (Neal *et al.*, 1982) and fibroblast (Harris *et al.*, 1981) as well as in fat tissues of rats (Kitabchi *et al.*, 1972). Therefore, in this study, the cytosolic fraction of liver tissue homogenates (100,000 g supernatant) obtained from healthy and STZ-induced diabetic rats was used to assess the potential inhibitory effect of HCQ on insulin degradation and to delineate the underlying mechanism of this interaction.

Diabetes mellitus, a chronic metabolic disease, is associated with several pathophysiological changes including alteration in microvascular permeability (Trap-Jensen, 1971; McMilllan, 1984), induction of an early increase in glomerular filtration (Mogensen *et al.*, 1971; Jenson *et al.*, 1981; Carney *et al.*, 1979; Craven *et al.*, 1987), alteration in drug metabolizing enzymes activities (Salmela *et al.*, 1980; Mahachai *et al.*, 1988; Zysset and Sommer, 1986; Skett and Joles, 1985), and change in blood concentration of lipoproteins and proteins (Kostner *et al.*, 1988; Miller *et al.*, 1990). All of these factors may potentially alter the pharmacokinetics and pharmacodynamics of drugs. Rheumatic patients may be predisposed towards the development of diabetes mellitus (Martin *et al.*, 1995; Brennan &. Feldmann, 1992). The co-existence of diabetes mellitus and rheumatoid arthritis may increase the risk of hypoglycemia where HCQ may sustain higher insulin levels in diabetic patients with some residual β-cell function.

In addition, rheumatic patients have many pathophysiological changes which may alter drug disposition. Pathophysiological changes, such as elevated α_1-acid glycoprotein concentrations (Belpaire *et al.*, 1989) and reduced cytochrome P-450 isoenzyme activity, which are often apparent in arthritic subjects, have been reported to influence the disposition kinetics of various drugs (Ferrari *et al.*, 1993; Belpair *et al.*, 1982; Schneider *et al.*, 1981). These alterations in drug disposition could have clinically significant pharmacodynamic effects. Therefore, the influence of arthritis and diabetes, for which HCQ is indicated, on the pharmacokinetics of this drug will be studied. Enantioselectivity in the pharmacokinetics of HCQ has been previously studied (McLachlan *et al.*, 1993; Ducharme *et al.*, 1995). Blood concentrations of R-HCQ exceeded those of the S enantiomer, whereas the metabolite concentrations of the S enantiomer were higher than those of R forms. This was attributed to slower urinary excretion and hepatic metabolism of the R enantiomer. Therefore, it is imperative to utilize stereospecific analytical assay as diseases may not equally affect the disposition of each enantiomer.

To study the suitability of the rat as an animal model of pharmacokinetic studies of HCQ enantiomers, the blood concentrations and urinary excretion of the enantiomers of HCQ will be examined in healthy rats after single i.v. and p.o. administration of racemic HCQ and will be qualitatively compared to humans.

Hydroxychloroquine does not bind to plasma proteins to a significant extent (Brocks *et al.*, 1994). Alteration in plasma protein binding, therefore, is not expected to have a significant impact on the pharmacokinetics of this drug. It is demonstrated that platelet counts are often elevated in patients with active arthritis (Farr *et al.*, 1983). The

degree of thrombocytosis appears to correlate with the extent of joint inflammation and with several acute-phase plasma proteins (Dixon *et al.*, 1983). In addition, a significant amount of drug in the blood circulation is bound to platelets (Brocks *et al*,, 1994). Therefore, platelet-rich plasma is a preferred medium, rather than platelet-poor plasma, to assess the effect of binding on the pharmacokinetics of this drug. As a result, the *in vitro* binding of HCQ enantiomers in healthy control and adjuvant-induced arthritic rats in platelet-poor plasma and platelet-rich plasma is also going to be determined.

4.2. Hypotheses

- Fasting serum glucose concentrations, insulin sensitivity and glucose tolerance in normal rats will not be affected during treatment with repeated oral administration of HCQ.
- Hydroxychloroquine improves glycemic control in diabetic rats in a concentration dependent manner.
- The improvement in glycemic control caused by HCQ is mainly due to the potential inhibitory effect of this drug on the insulin degrading activity of the cytosolic fraction of some tissue homogenates particularly liver tissue.

- The pharmacokinetics of HCQ enantiomers are altered in adjuvant arthritis in rats due to:

  a. Decreased intrinsic metabolism

  b. Decreased free concentration

- The pharmacokinetics of HCQ enantiomers are altered in experimental diabetes mellitus due to:
    a. Increased intrinsic metabolism
    b. Increased renal clearance
- Diabetes- and arthritis-induced drug disposition changes are stereoselective.
- Rat is a suitable animal to study diabetes and inflammation induced alterations in disposition of HCQ enantiomers.

4.3. Specific Objectives

- To study the effect of chronic administration of HCQ on fasting serum glucose, insulin sensitivity and glucose tolerance in healthy rats.
- To study the effect of chronic administration of HCQ on glycemic profiles (percent change in glucose and insulin from baseline *vs* time curve) following exogenous administration of rapid acting insulin in STZ-induced diabetic rats.
- To determine the relationship between various doses of HCQ administered and its blood concentrations on improvement in glycemic control in STZ-induced diabetic rats.
- To determine the underlying mechanism of HCQ action on insulin degradation in cytosolic fraction of liver homogenates from healthy and STZ-induced diabetic rats.
- To investigate the effect of STZ-induced diabetes and adjuvant-induced arthritis on the disposition of HCQ enantiomers.

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Chapter 2

INSULIN- SPARING EFFECT OF HYDROXYCHLOROQUINE IN HEALTHY AND DIABETIC RATS*

INTRODUCTION

Hydroxychloroquine (HCQ) is an antimalarial drug that also acts as a disease-modifying agent in rheumatoid arthritis and lupus erythematosus (HERA group, 1995; Ruzicka *et al.*, 1992). Attention has recently focused on its effect on glucose homeostasis in patients with hyperglycemia due to diabetes. A small placebo controlled trial of this drug in 38 patients with non-insulin dependent diabetes mellitus (NIDDM) resistant to commonly used therapy demonstrated a dramatic improvement in glucose and glycated hemoglobin following six months of therapy with this drug (Quatraro *et al.*, 1990). A much larger study of 135 patients (Grestein *et al.*, 1997) also showed that HCQ significantly improved glycemic control in patients with NIDDM. These trials suggest that this drug may be therapeutically useful for the treatment of NIDDM. Despite these clinical data, little information is available regarding the mechanism by which HCQ affects glucose and insulin metabolism. Data describing the action of the parent compound chloroquine, which is structurally related to HCQ, suggests that chloroquine reduces intracellular insulin degradation and increases its accumulation in isolated rat adipocytes (Hammons *et al.*, 1980; Marshall *et al.*, 1980), mouse fibroblasts (Knutson *et al.*, 1985), rat hepatocytes (Khan *et al.*, 1985; Hoffman *et al.*, 1980), and rat kidney (Thomas *et al.*,

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1987). Chloroquine also slows insulin receptor recycling (Smith *et al.*, 1989) and stimulates insulin-mediated glucose transfer (Cynober *et al.*, 1987).

This study was designed to investigate the hypoglycemic effects of HCQ in both healthy and diabetic animals and to elucidate the mechanism by which it may exert its effect. The blood HCQ concentration-effect relationships were also assessed in order to explore the possibility of providing a more rationale approach to diabetes.

MATERIALS AND METHODS

Chemical and Reagents

Streptozotocin (STZ), racemic chloroquine diphosphate, and ortho-toluidine (Sigma Chemical Co. St. Louis, MO), regular porcine insulin (Iletin II, Eli Lilly and Company, Indianapolis, IN), racemic HCQ sulfate (gift from Sepracor Inc., Marborough, MA), (+)-di-O-acetyl-L-tartaric anhydride (Fluka Chemika, Buchs, Switzerland). All other reagents were analytical grade and available commercially.

Animals

Adult male Sprague-Dawley rats weighing between 300 and 350 g were housed in groups of two in separate cages with free access to standard laboratory food and water. Experiments were performed on healthy and STZ-induced diabetic rats.

Healthy Rats

Healthy rats received either normal saline (n = 5) or 160 mg/kg/day of HCQ (n = 5) orally for ten days. Prior to, and ten days after administration of HCQ, fasting plasma glucose concentrations were measured. All animals subsequently underwent i.v. insulin challenge (0.25 U/kg) and plasma samples were collected 10 min before and every

15 min thereafter over a 1.5-h period. On the next day, glucose tolerance test (1g/kg, i.v.) was performed and plasma samples were collected every 5 min over a 0.8-h period.

STZ-induced Diabetic Animals

Insulin-dependent diabetes mellitus (IDDM) was induced by a single i.p. injection of 60 mg/kg of STZ. The solution of STZ was prepared immediately before injection by dissolving the powder in 0.5 ml of 0.1 M citrate buffer (pH 4.2). Rats were considered diabetic and included in the study if their serum glucose was greater than 300 mg/dL. Diabetic rats received their HCQ treatment ten days after induction of diabetes (Mehvar *et al.*, 1991).

All diabetic animals were matched for their initial body weight and divided into four groups (n = 7) which included a control (C) and three test groups. Control animals were administered normal saline whereas test animals received 80 (T80), 120 (T120) or 160 (T160) mg /kg doses of racemic HCQ dissolved in distilled water (1 mL) administered once a day, for 10 days, by gastric gavage.

One day prior to insulin administration, under methoxyflurane anesthesia (Metofane, Pitman-Moore, Washington Crossing, NJ) the right jugular vein of the animals was canulated with silastic tubing (Dow Corning, Midland, MI) to permit dosing and sample collection. All rats underwent an i.v. insulin test (0.1 U/kg) after 10 days of treatment. The tests were performed after an overnight fast of 16 h, during which the rats had free access to water. Blood samples (0.2 mL) were collected through the jugular vein canula and centrifuged in a microcentrifuge (model 235C, Fisher Scientific, Itasca, IL) for 5 min, for subsequent plasma collection. Samples were collected 30 minutes before the

injection of insulin (baseline) and every 15 min over a 3-hr period to measure glucose and insulin levels. The samples were then frozen at -20°C until analysis.

Measurement of Glucose and Insulin in Plasma

Plasma glucose concentrations were determined using the ortho-toluidine method (Linne *et al.*, 1979). To 50 µL of plasma was added 3 mL of ortho-toluidine reagent and the mixture incubated for 12 min in a boiling water bath. Samples were then cooled in an ice bath for 5 min and left at room temperature for another10 min. The color intensity was read at 630 nm. The color was found to be stable for at least one hour.

Plasma insulin concentrations were measured at the Muttart Diabetes Centre (University of Alberta) using a validated routine double-antibody radioimmunoassay (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden).

Hydroxychloroquine Assay

Concentrations of HCQ enantiomers in blood were measured using a previously described HPLC method (Brocks *et al.*, 1992). Briefly, after addition of racemic-chloroquine (internal standard) blood samples (0.2 mL) were alkalinized and extracted with 5 mL of diethyl ether. After solvent evaporation the residues were derivatized with a solution of (+)-di-O-acetyl-L-tartaric anhydride at 45°C for 30 min. The resulting diastereomers were then resolved using a C8 analytical column with a mobile phase consisting of 0.05 M KH_2PO_4 (pH 3), methanol-ethanol-triethylamine (80:20:1:0.08). The UV detection wavelength was set at 343 nm.

Data Analysis

The percent change from baseline (from t=0 minutes) in both glucose and insulin was calculated at each time point after i.v. injection of insulin and plotted. The area under the curve for both the percent change in glucose and the percent change in insulin was estimated by the trapezoidal rule. Percent maximum reduction in serum glucose (MRG), area under the percent reduction in glucose-time profile (AUG), and area under the percent increase in serum insulin-time profile (AUI) were selected as pharmacodynamic endpoints. The associations between HCQ concentrations or dose and the changes in glucose and insulin were examined using linear regression. Differences between two means were assessed using the Student's t-test for unpaired data. The differences among the groups were determined based on a single factor analysis of variance (ANOVA) followed by the Duncan's multiple range test. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

Healthy Rats

Hydroxychloroquine treatment did not influence fasting plasma glucose concentrations (98.26 ± 2.1 *vs* 97.76 ± 4.1 mg/dL). Following injection of insulin, control and HCQ treated rats showed no significant differences in the area under the glucose concentration *vs* time profiles (55.68 ± 6.5 *vs* 48.28 ± 4.3, mg.h/dL) (Fig. 2-1A). Similarly, after glucose injection the area under the plasma glucose concentration *vs* time profiles was not significantly affected by 10 days of HCQ treatment (147.7 ± 6.1 *vs* 147.4 ± 6.2, mg.h/dL) (Fig. 2-1B).

STZ-induced Diabetic Rats

There was no significant weight gain or loss following induction and repeated administration of HCQ (Table 2-1).

Table 2-1. Body weight of different groups of diabetic rats (n = 7 for each group).

Body weight (g)[a]	Control	HCQ treated		
	C	T80[b]	T120[c]	T160[d]
Before induction of diabetes	315 ± 6.3	317 ± 6.2	302 ± 6.8	300 ± 5.4
10 days after treatment with HCQ	297 ± 6.7	299 ± 5.8	274 ± 12.4	277 ± 9.4

[a] No intra- or inter-group differences.
[b] HCQ-treated diabetic rats (80 mg/kg/day, for 10 days)
[c] HCQ-treated diabetic rats (120 mg/kg/day, for 10 days)
[d] HCQ-treated diabetic rats (160 mg/kg/day, for 10 days)

Diabetic control and diabetic HCQ-treated rats challenged with insulin showed significant intra-group differences in MRG and AUG (Fig. 2-2). T160 rats had greater MRG and AUG (34.1± 3.6 %, 76.5 ± 10.9 %•t, respectively) values as compared to those observed in T120 (24.3 ± 2.1 % and 46.2 ± 7.4, %•t), T80 (17.1± 0.7 % and 37.4 ± 3.8, %•t), and control rats (14.7 ± 0.94 % and 26.3 ± 2.7, %•t). MRG and AUG were significantly increased in the T120 rats compared to control. T80 treated rats did not show any significant differences as compared with control diabetic rats. Least-squares linear regression analysis of the relationship between effect and HCQ blood concentration and dose was also performed. There was a significant linear relationship between the effect of HCQ on glucose reduction and HCQ concentration (AUG, $r = 0.86$, $P < 0.001$; Fig. 2-3A) and HCQ dose ($r = 0.65$, $P < 0.002$; Fig. 2-3B). Hydroxychloroquine-treated diabetic rats challenged with insulin showed improvement in serum insulin-time profiles, as

measured by AUI. The percent increase in insulin levels following injection of 0.1 U/kg body weight of insulin in the T120 group is shown in figure 2-4A. We tested the time-course of insulin in only two rats in each of T80 and T160 and six rats in T120 groups. The AUI values were much greater in T160 (187.5 and 350, %•t; n=2) and T120 (143.4 ± 25.2, %•t; n = 6) as compared to those observed in T80 (45.8 and 51.5, %•t; n = 2). When all data generated from the three administered doses were pooled a significant linear relationship between the AUI and HCQ blood concentrations ($r = 0.71$, $P < 0.05$; fig. 2-4B) was observed.

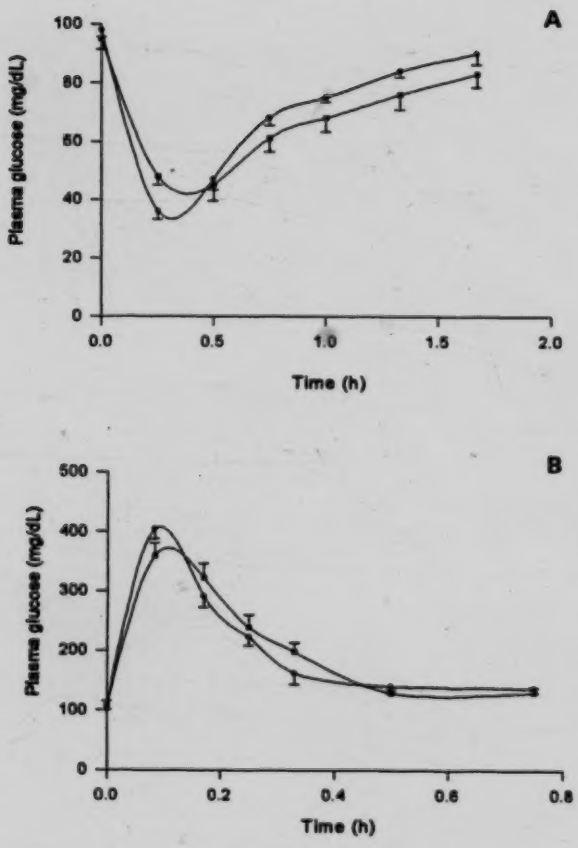

Figure 2-1. (A) Plasma glucose concentration-time profile in response to i.v. insulin (0.25 U/kg) in non-diabetic control rats (●) and non-diabetic HCQ-treated rats (160 mg/kg/day) for 10 days (■). **(B)** Plasma glucose concentration-time profile in response to i.v. glucose (1 g/kg) in non-diabetic control rats (●) and non-diabetic HCQ-treated rats (160 mg/kg/day, for 10 days) (■). n =5 rats in each group. Values are means with SEM bars.

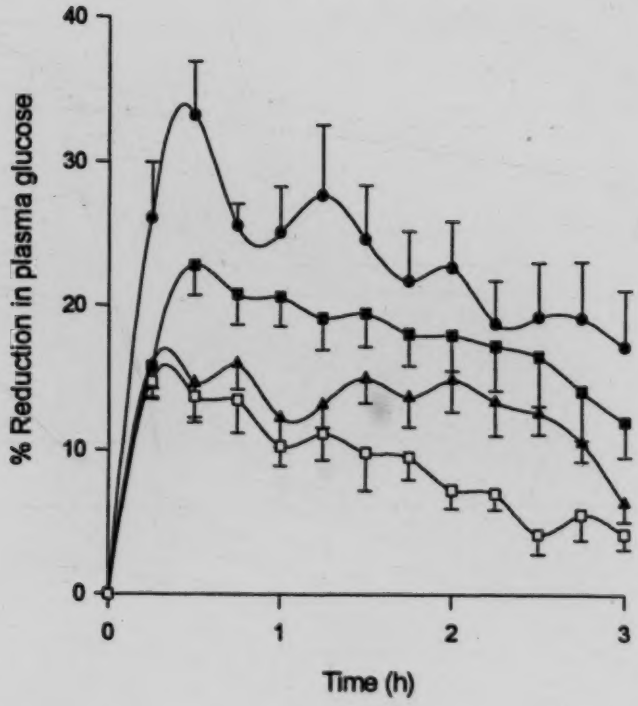

Figure 2-2. Percent reduction in plasma glucose from baseline vs time profile in control (□) and HCQ-treated diabetic rats with 80 mg/kg/day (▲), 120 mg/kg/day (■) and 160 mg/kg/day (●) for 10 days in response to i.v. administration of insulin (0.1 U/kg). n = 7 rats in each group; Values are mean with SEM bars.

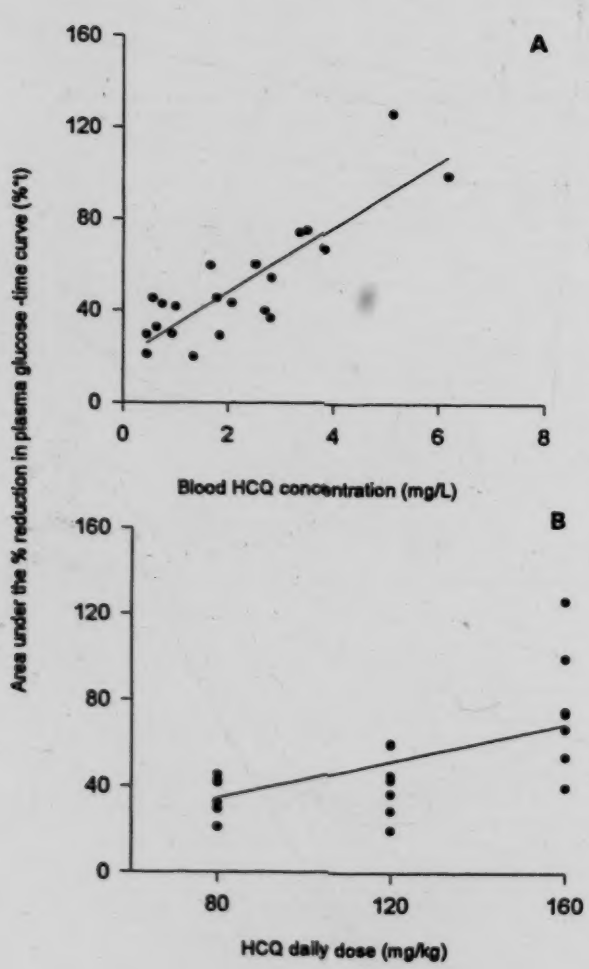

Figure 2-3. Area under the percent reduction in plasma glucose from baseline *vs* (A) blood trough HCQ concentrations following repeated oral administration of various doses of HCQ for 10 days and (B) HCQ doses.

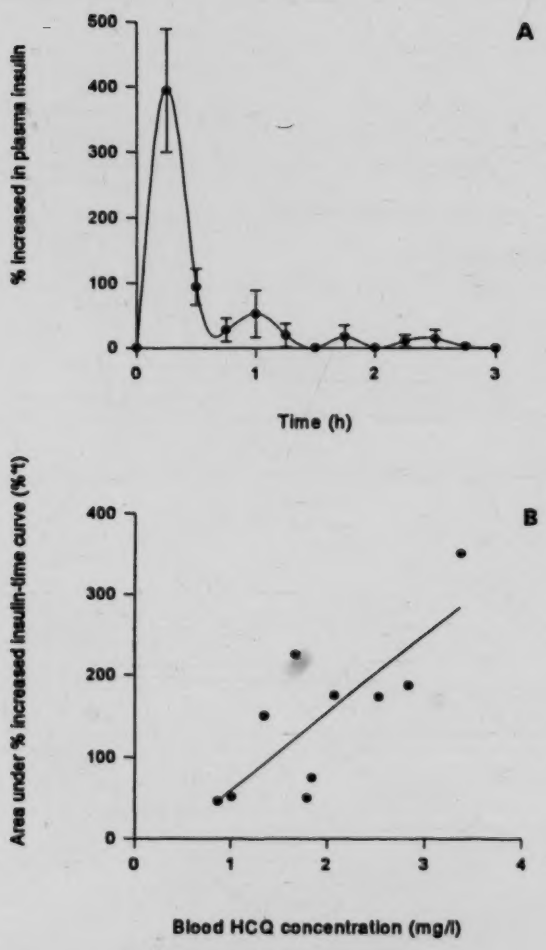

Figure 2-4. (A.) Percent increase in plasma insulin from baseline *vs* time profile in HCQ-treated diabetic rats with 120 mg/kg/day for 10 days in response to i.v. administration of insulin (0.1U/kg). (B). Area under the percent increase in plasma insulin from baseline *vs* blood trough HCQ concentrations following repeated oral administration of various doses of HCQ.

DISCUSSION

Our data suggest that in healthy rats, HCQ does not influence plasma glucose concentrations, insulin sensitivity, or tolerance to a glucose load in healthy rats. It is possible that in non-diabetic animals the normal homeostatic mechanisms responsible for insulin-glucose regulation, including decreased insulin concentrations and/or a counter-regulatory increase in the levels of cortisol, glucagon, growth hormone, and catecholamines, may compensate for possible HCQ-induced reduction in insulin metabolism. Our data on the effect of HCQ on glucose homeostasis is consistent with the lack of effect of chloroquine on serum glucose and insulin concentrations in humans and rats (Ericsson *et al.*, 1981; Smith *et al.*, 1987; Okitolanda *et al.*, 1984). Chloroquine does not appear to have any effect on insulin or glucose metabolism in healthy humans (Smith *et al.*, 1987; Phillips *et al.*, 1986).

The data for STZ-induced diabetic animals clearly show that the HCQ treatment caused a significant decrease in glucose and a significant increase in insulin profiles as reflected in the lower plasma glucose and higher plasma insulin levels following i.v. insulin challenge.

The mechanism responsible for the possible beneficial effects of HCQ on glucose homoeostasis is not clear. *In vitro* studies suggest that chloroquine may reduce intracellular insulin degradation at the postreceptor level and slow receptor recycling resulting in intracellular insulin accumulation (Smith *et al.*, 1989). As an acidotrophic agent, chloroquine, concentrates in endosomes which may result in inhibition of endosomal degradation of insulin internalized with its receptor (Varandani *et al.*, 1982;

Smith *et al.*, 1989). Accumulation of chloroquine in endososomes causes an increase in pH which may then lead to inactivation of proteolytic enzymes (De Duve *et al.*, 1974; Smith *et al.*, 1989). The drug also appears to enhance binding of insulin to its membrane receptor in lymphocytes (Iwamoto *et al.*, 1981). However, some *in vitro* studies have failed to demonstrate an effect of chloroquine on insulin-mediated glucose transport. For example, in adipocytes chloroquine does not affect stimulation of glucose transport by insulin (Hammons and Jarett, 1980; Marshal and Olefsky, 1980). In hepatocytes, the insulin-mediated stimulation of glucose incorporation into glycogen is somewhat impaired by chloroquine (Hofman *et al.*, 1980). In contrast to these findings, the stimulatory effect of chloroquine on insulin-mediated glucose transport was observed by Cynober *in vitro* (Cynober *et al.*, 1987) and Blazer *in vivo* (Blazer *et al.*, 1984). One possible explanation for the lack of effect of chloroquine on insulin-mediated glucose transport in some aforementioned *in vitro* studies is that the drug was incubated with the cells for a short period of time (15 min). Longer exposure (3h) of the cells to insulin and chloroquine would be required to observe the effect of the hormone on glucose uptake (Cynober *et al.*, 1987). Theoretically, HCQ should behave in a manner similar to chloroquine. In our study, following administration of insulin to HCQ-treated diabetic rats, a significant increase in serum insulin levels was observed. Therefore, the subsequent improvement in glycemic control may have been mediated by the effect of insulin on cellular glucose transport. Our *in vitro* studies on the effect of HCQ on insulin degradation (chapter 3) clearly indicated that insulin metabolism was inhibited in the cytosolic fraction of rat liver homogenates containig insulin protease.

Hydroxychloroquine may affect serum insulin concentrations by increasing secretion from pancreatic β-cells. This should result in increased fasting serum insulin in diabetic rats with some β-cell residual functions. However, the insulin secretory function of the pancreas in our diabetic rats, as indicated by low levels of fasting plasma insulin, was drastically reduced. In addition, in order to evaluate the effect of HCQ on glucose homoeostasis, insulin had to be administered exogenously to the rats. It is therefore likely that clearance of insulin is reduced as a result of HCQ treatment. Measurement of C-peptide, which is secreted along with insulin from the pancreatic β-cells and totally excreted unchanged in the urine, seems to be a good index for insulin secretion (Polonsky and Rubenstein, 1984). Renal clearance of this peptide is assumed to remain unchanged after antimalarial treatment (Landewee *et al.*, 1995). The improvement in glycemic control observed in patients with NIDDM who were on chloroquine seems to be due to decreased insulin degradation rather than increased pancreatic insulin secretion. This is evident by unchanged C-peptide levels in normal subjects and in patients with rheumatoid arthritis treated with chloroquine (Ericsson, *et al.*, 1981; Smith *et al.*, 1989). Collectively, these results suggest that HCQ may reduce the clearance of insulin *in vivo*.

The insulin sensitivity test used in this study might provide a good estimate of the overall responsiveness to this hormone in target tissues. Insulin clearance is a receptor-mediated process and shows saturation at high serum insulin levels. Thus, when target tissues are exposed to insulin concentrations greater than 300 μU/mL (Polonsky and *et al.* 1983; Katz and Rubenstein, 1973), it is difficult to compare the activity of insulin on glucose homeostasis. Intravenous administration of 0.1 U/kg gives rise to a maximum

concentration of less than 100 μU/mL at which saturation of insulin metabolism is very unlikely. Moreover, the metabolic clearance rate of ^{125}I-insulin was shown to be significantly elevated due, in part, to increased binding of insulin to a specific binding receptor in diabetic animals (Philippe *et al.*, 1981). This could also be due to an increased number of insulin receptors on liver plasma membrane (Davidson *et al*, 1977; Vigneri, *et al.*, 1978) and other target tissue membranes (Rabkin *et al.*, 1986 ; Kobayashi *et al.*, 1979).

It is important to understand the concentration-effect relationship in the body leading to prediction of drug effects in subjects. A high degree of correlation was found between AUG and HCQ blood concentrations and dose. The correlation of AUI with HCQ blood concentrations also showed a significant linear relationship. However, such linearity seemed to be less noticeable than that of AUG and HCQ blood levels due to the smaller number of data points used to perform this correlation. Whether or not the AUG is a better predictor of effect of HCQ on glycemic control than AUI can't be determined from these data. Correlations for all respective measured effects seemed to be better when HCQ concentrations, rather than dose were given.

Insulin resistance, a common feature of diabetes mellitus, has been attributed to several factors. Only in rare cases has insulin resistance in patients with NIDDM been ascribed to abnormal islet cell products or circulating insulin antagonists (Smith *et al.*, 1987). In most cases the insulin resistance found in patients with NIDDM has been attributed to pre-receptor and post-receptor defects. However, this assumption has recently been challenged by demonstrating normal insulin binding to hepatic membranes

isolated from diabetic patients. Thus, a postbinding defect in insulin action must be responsible for the observed insulin resistance in patients with well-established hyperglycemia (Arner *et al.*, 1986). Interestingly, it has been shown that some subjects with insulin resistance, including patients with NIDDM, exhibit an increased rate of intracellular insulin degradation (Vasquez *et al.*, 1985). This may imply that HCQ is partially able to overcome the post-receptor abnormality responsible for insulin resistance in patients with NIDDM. Therefore, the increased postreceptor insulin levels which enhance the metabolic effects of hormone and accelerate the insulin-stimulated glucose transport (Cynober *et al.*, 1987 ; Quatraro *et al.*, 1990) may lead to better management of the disease. Evidence also indicates that in the insulin-stimulated state, muscle is the primary tissue responsible for the insulin resistance (DeFronzo, 1986). Therefore, increased glucose utilization in the periphery may account for the beneficial effect of HCQ in glucose homeostasis in diabetes mellitus. In addition, the possible effect of HCQ on stimulation of gluconeogenesis at the hepatic level should not be ignored. Hydroxychloroquine may sustain higher insulin levels and therefore have therapeutic potential in the treatment of patients who have some residual β-cell function. This drug may also be used for the treatment of insulin resistant diabetes due to accelerated insulin degradation activity in patients with IDDM.

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Chapter 3

EFFECT OF HYDROXYCHLOROQUINE ON INSULIN METABOLISM IN CYTOSOLIC FRACTION OF LIVER HOMOGENATES FROM HEALTHY AND DIABETIC RATS

INTRODUCTION

Hydroxychloroquine (HCQ) is an antimalarial drug increasingly being used as a disease-modifying agent in rheumatoid arthritis (The HERA study group, 1995; Clark *et al.*, 1993). Of particular interest to this study are reports linking HCQ and chloroquine, another antimalarial drug with a physicochemical similarity to HCQ, to glucose and insulin homeostasis. Numerous studies have used chloroquine as a tool to study binding and insulin metabolism in target tissues. *In vitro* evidence has shown that chloroquine reduces intracellular insulin degradation, increases intracellular insulin accumulation, slows receptor recycling and stimulates insulin-mediated glucose transport (Smith *et al.*, 1989; Cynober *et al.*, 1987). Case reports and clinical studies suggest that chloroquine improves glucose tolerance in patients with non-insulin dependent diabetes mellitus (NIDDM) and insulin resistance syndromes (Blazar *et al.*, 1984 ; Smith *et al.*, 1987).

Literature review on the effects of HCQ on glucose homeostasis reveals a single preliminary clinical trial which demonstrated improved glycemic control in patients with NIDDM resistant to standard therapy (Quatraro *et al.*, 1988). Very recently a much larger study with 135 patients with NIDDM was brought to our attention. This study also showed that HCQ significantly improved glycemic control in these patients (Gerstein *et al.*, 1997). We have recently demonstrated that HCQ reduces insulin clearance and

consequently glucose levels in a concentration dependent manner in streptozotocin (STZ)-induced diabetic rats (Emami *et al.*, 1995). In healthy rats, however, glucose tolerance and insulin sensitivity did not differ between control and HCQ-treated animals following challenge with i.v. administered glucose or insulin (Emami *et al.*, 1995). However, as yet the underlying mechanism(s) of the effects of HCQ on glucose and insulin metabolism has not been elucidated. Additionally, HCQ may have several advantages over chloroquine due to lower frequency and severity of retinal and gastrointestinal toxicities (Finbloom *et al.*, 1985; Tett *et al.*, 1990). Furthermore, the manufacturer has removed chloroquine's indication as an antirheumatic agent due to the risk of ocular toxicity (Salmeron and Lipsky, 1983).

In view of these observations, we were prompted to investigate the potential inhibitory effect of HCQ on insulin degrading activity of the cytosolic fraction of liver homogenates obtained from healthy and STZ-induced diabetic rats. It is possible that the effect of HCQ on insulin and glucose homeostasis might be, in part, enzyme mediated processes, most likely via insulin protease.

MATERIALS AND METHODS

Chemical and Reagents

Streptozotocin and ortho-toluidine (Sigma Chemical Co. St. Louis, MO), crystalline and ^{125}I-labelled porcine insulin (ICN Pharmaceuticals Canada Ltd). Racemic HCQ sulfate (rac-HCQ) was a gift from Sepracor Inc. (Marborough, MA). All other reagents were analytical grade and commercially available.

Animals

Adult male Sprague-Dawley rats weighing between 300 and 350 g were kept in metabolic cages with free access to standard laboratory food and water. Experiments were performed using the cytosolic fraction of liver homogenates from healthy or STZ-induced diabetic rats.

Insulin-dependent diabetes mellitus (IDDM) was induced by a single i.p. injection of 60 mg/kg of STZ. The solution of STZ was prepared immediately before injection by dissolving the powder in 0.5 ml of 0.1 M citrate buffer (pH 4.2). Rats were considered diabetic, and included in the study, if their serum glucose was greater than 300 mg/dL.

Preparation of Cytosolic Fraction of Liver Homogenates

Animals were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, NJ) and, after opening the abdominal cavity, the hepatic portal vein was catheterized with a 16-gauge angiocatheter to serve as an inlet. The thoracic inferior vena cava was cut and the liver was perfused with cold 0.9% NaCl to remove residual blood. The liver was then immediately excised and placed in 4°C sucrose solution. All five lobes of the liver were harvested, cut into small pieces, gently blotted to remove excess liquid, weighed, and minced with scissors. The tissues were homogenized in a smooth-walled homogenizing tube with a teflon pestle (Potter Elvehjem tissue grinder, Talboys Engineering, Montrose, PA) in 0.33 M sucrose solution. The volumes of homogenates were adjusted to 2.5% W/V and centrifuged at 100,000 g for 90 min. At the end of centrifugation the pellet was discarded and the supernatant (cytosolic extract) was used in the insulin degradation studies (Neal *et al.* 1982).

Enzyme Kinetics, Insulin Degrading Activity and Inhibition Studies

The degradative activity of the 100,000 g supernatant of the liver homogenates was determined by measuring the production of trichloroacetic acid (TCA)-soluble radioactivity from ^{125}I-insulin. The incubation mixtures contained pH 7.4 Krebs-Henseleit phosphate buffer (KHB) with 0.3% bovine serum albumin (BSA). Solutions of insulin were prepared in a potassium phosphate buffer (pH 7.5) containing 0.5% BSA. The substrate, inhibitors, and incubation medium were preincubated at 37°C for 15 min and the degradation activity was initiated by the addition of various concentrations of insulin. The substrates, ^{125}I-insulin (0.5-15 ng/mL) and native insulin (1-30 μg/mL), were incubated with the cytosolic liver extract (0.4 mg/mL) in the presence and absence of HCQ (5 mg/mL) at a final volume of 100 μL. To terminate the degradation an equal volume of cold 10% TCA solution was added to the appropriate test tubes. The tubes were removed from the 37°C water bath, thoroughly mixed, and left on an ice bath for 15 min. The precipitate formed was separated from the supernatant by centrifuging at 3000 rpm for 15 min, dissolved in 30% KOH, and diluted to a volume equal to that of the supernatant solution. The radioactivity in the supernatant and precipitate were counted in a Gamma Scintillation Counter (Gamma 8000, Beckman Instrument, Inc., CA). The percent degraded was calculated from the increase in TCA-soluble radioactivity in tubes containing hepatic cytosolic extract above that observed in control tubes incubated without liver extract. The amount of cytosolic extract used in the degradation studies was selected so that degradation of substrate followed a first order kinetic reaction over the 7 minutes period of the assay. The 7 min degradation time was selected from the linear

portion of the insulin degradation time course at 37°C by the cytosolic fraction of liver homogenates.

Time Course of Insulin Degradation

Degradation of insulin was measured at a concentration of 1 μg/mL at 37°C. The test tubes containing cytosolic fractions of liver homogenates and insulin, in KHB containing BSA, were shaken and transferred to a 37°C water bath. The test tubes were kept in the water bath for a period of time varying from 1 to 45 minutes. At the end of each time interval an equal volume (100 μL) of 10% TCA was added to the appropriate tubes which were then removed from the water bath. The percent insulin degradation was determined as previously described above. The proper incubation time, used to determine the initial degradation rate of insulin, was obtained from the linear portion of the resultant time-profile curve.

Effect of HCQ on the Degradation of Insulin

Degradation studies to examine the effect of rac-HCQ on the insulin degradation activity of the cytosolic fraction of liver homogenates were performed in the following manner: 20 μL of 100,000 g supernatant (40 μg protein) with 10 μL of substrate composed of ^{125}I-insulin (0.05 ng) and native insulin (0.1 μg) in the presence of 2.5, 5, 10, 20, and 40 mg/ml of HCQ, were incubated for 7 min in KHB containing BSA (pH 7.4). The concentration of insulin for the inhibition study was selected from the linear portion of the concentration-rate plot (Fig. 3-2).

Analytical Methods

Serum glucose concentrations were determined using the ortho-toluidine method (Linne *et al.* 1979). The protein content of the cytosolic liver extract was determined according to the Lowry method using BSA as a standard (Lowry *et al.* 1951). The ^{125}I was counted in a Beckman Gamma 8000 Scintillation Counter.

Data Analysis

All reaction velocities were based on a 7-min incubation which falls within the linear time period of the reaction. The Michaelis-Menten kinetic parameters of insulin degradation were obtained by fitting the reaction velocities by using the following equation in the nonlinear least-square regression analysis program Win-nonlin (Scientific Consulting, Inc. Apex, North Carolina)

$$V = V_{max} \cdot C_s / K_m + C_s$$

The affinity constant K_m, is the substrate concentration at which the reaction velocity equals 50% of V_{max}, V is the degradation rate, V_{max} is the maximum degradation rate, and C is the concentration of substrate. The kinetic parameters were estimated initially by graphical analysis of a Hanes plot (substrate concentration/degradation rate against substrate concentration) and the values obtained were then used as first estimates for non-linear least-squares regression analysis.

Statistical differences between two means were assessed using the Student's t-test for unpaired data. Differences were considered significant at $P < 0.05$. Data are presented as mean ± S.D.

RESULTS

Time Course of Insulin Degradation

The time course of insulin degradation at 37°C by the cytosolic fraction of liver homogenates is illustrated in figure 3-1A. Degradation of insulin was linear during the first 10-minute incubation period. Thus, 7 minutes of degradation time was used, hereafter, to determine the initial degradation rate of insulin.

Effect of HCQ on Insulin Degradation

Figure 3-1B depicts the inhibition of insulin degradation by rac-HCQ at a concentration of 1 μg/mL of insulin using cytosolic fraction of liver homogenates from healthy and diabetic rats. Racemic HCQ inhibited cytosolic degradation of insulin in a concentration-dependent manner. Incubation of insulin with various concentrations of HCQ (2.5-40 mg/mL) significantly reduced the rate of insulin degradation. The concentration of 5 mg/mL of HCQ was selected from the linear portion of the curves to further study the kinetics and to characterize the various kinetic parameters of insulin metabolism by HCQ.

Concentration Dependency of Insulin Degradation

The reaction velocities *vs* substrate concentrations with and without rac-HCQ, using cytosolic fraction of liver homogenates, from both healthy and diabetic rats, are depicted in figure 3-2. These plots were consistent with a sigmoid V_{max} model. The estimated kinetic parameters V_{max}, K_m, and V_{max} / K_m are listed in table 3-1. Significant differences were observed for V_{max}, and V_{max} / K_m in the presence of rac-HCQ using cytosolic extract from healthy and diabetic animals. Insulin metabolism was significantly

reduced in insulin-deficient rats compared to healthy animals (Table 3-1; Figs 3-1B, 3-2). Eadie-Hofstee plots (reaction velocity *vs* velocity over substrate concentrations, Fig. 3-3) were constructed to assess the multiplicity of enzymes involved in degradation of insulin. Since the hepatic cytosolic fraction of both healthy and diabetic rats gave a monophasic relationship ($r = 0.98$) only a one-component enzyme system is involved in insulin metabolism by cytosolic fraction of rat liver homogenates.

Table 3-1. Kinetic parameters of insulin metabolism using cytosolic fraction of liver from healthy and diabetic rats in the presence and absence of HCQ.

	Healthy		Diabetic	
Parameters	**Control**	**HCQ**	**Control**	**HCQ**
V_{max} (ng/min/mg protein)	3.63 ± 0.46	1.97 ± 0.13[a]	0.718 ± 0.06[b]	0.360 ± 0.024[a,b]
K_m (μg/ml)	13.81 ± 2.56	17.50 ± 1.73	14.03 ± 1.46	15.75 ± 1.71
V_{max} / K_m (ml/g/min)	0.265 ± 0.015	0.112 ± 0.004[a]	0.05 ± 0.002[b]	0.023 ± 0.001[a,b]

[a] Significantly different from respective control ($P < 0.001$).
[b] Significantly different from healthy group ($P < 0.001$)

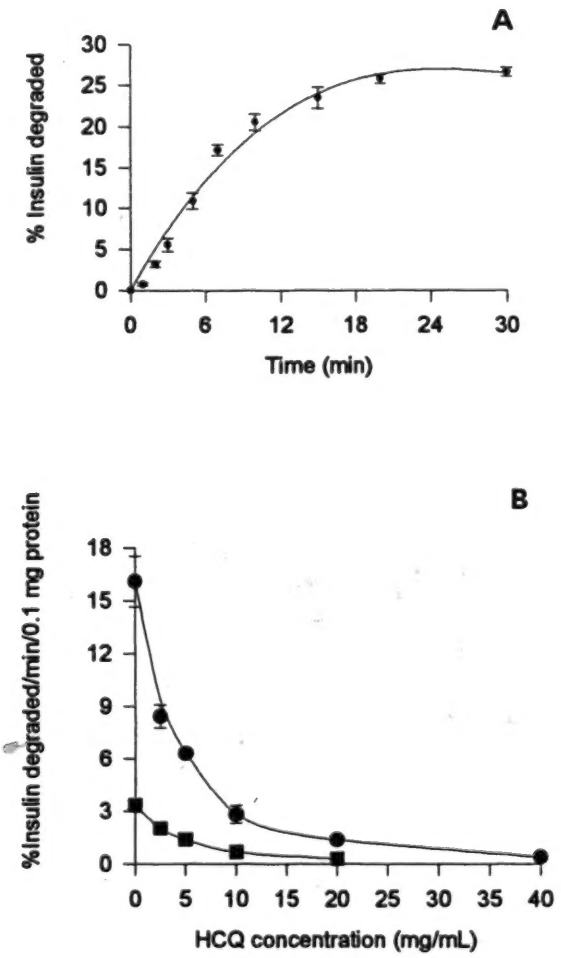

Figure 3-1. (A) Time course of insulin degradation by cytosolic preparation of rat liver homogenates at 37°C. Degradation was measured at an insulin concentration of 1 μg/mL. Each point represents the mean ± S.D. of four experiments. (B) Effect of various concentrations of rac-HCQ on insulin degradation by cytosolic preparation of liver homogenates of healthy (●) and diabetic (■) rats at 37°C. Degradation was measured at an insulin concentration of 1 μg/L. Each point represents the mean ± S.D. of six experiments.

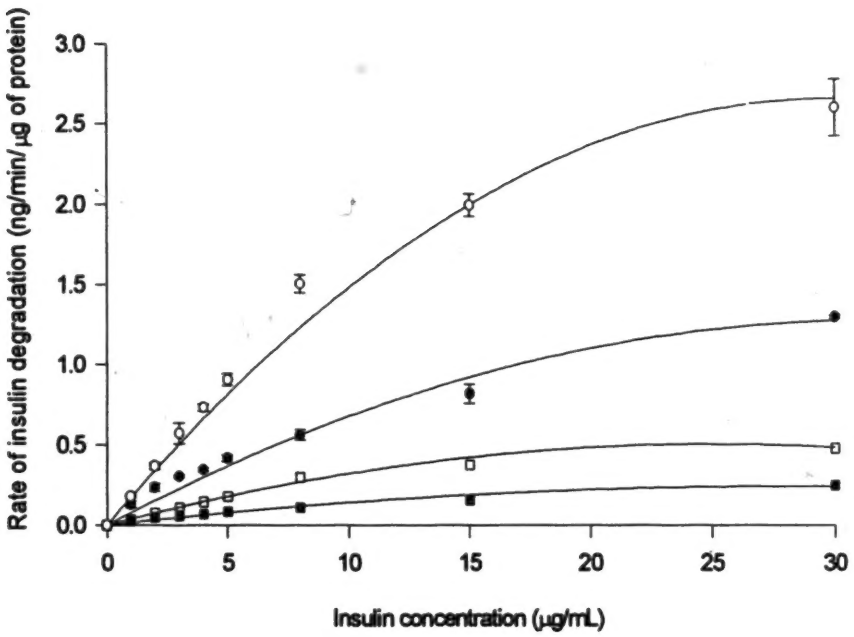

Figure 3-2. Concentration dependence of the degradation rate of insulin by cytosolic preparation of rat livers. Initial degradation rate of insulin was measured in the concentration range from 1-30 μg/mL. Uptake was initiated by the addition of various concentrations of insulin. Insulin degradation was measured at 37°C and pH 7.4 over 7 min in the incubation medium (for composition, see text). Cytosolic preparations obtained from healthy animals in the absence of HCQ (○) and presence of 5 mg/mL rac-HCQ (●); cytosolic preparation obtained from diabetic animals in the absence of HCQ (□) and in the presence of 5 mg/mL rac-HCQ (■). Each point represents the mean ± S.D. of four rats.

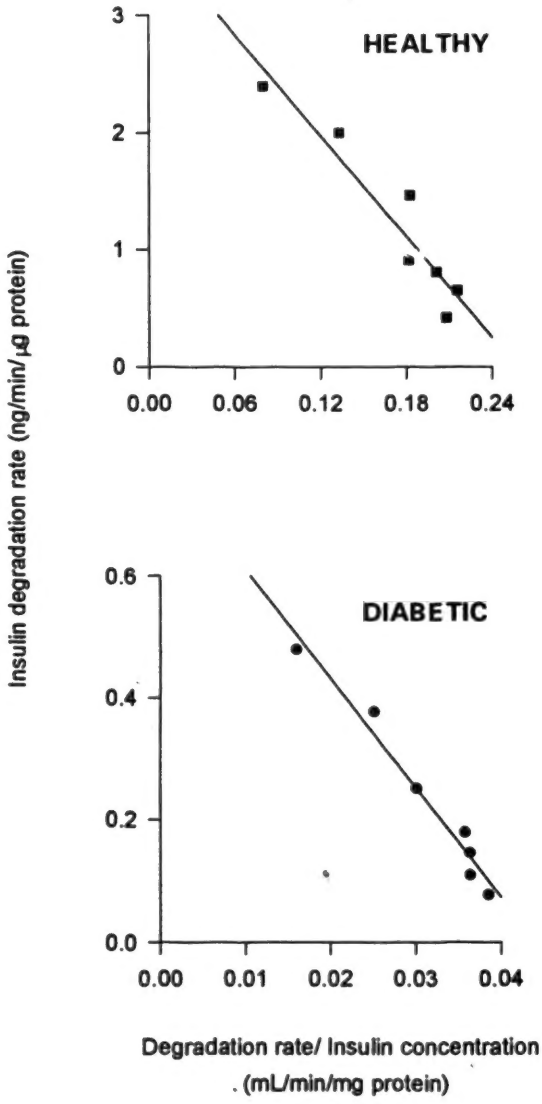

Figure 3-3. Eadie-Hofstee plots of insulin degradation in cytosolic fraction of rat livers. Each point represents the mean ± S.D. of four rats.

DISCUSSION AND CONCLUSION

More than 50% of insulin is cleared in a single pass through the liver (Smith *et al.* 1989). Insulin degradation is a complex process which has not been completely elucidated. The initial step in insulin degradation is binding to the cell membrane (Terris *et al.* 1975). Insulin uptake is mediated by specific insulin receptors in a number of tissues including liver. Once the insulin-receptor complex has been internalized, insulin undergoes rapid degradation (Smith *et al.* 1989). Some insulin is, however, degraded on the cell membrane in absence of internalization, and is metabolized by membrane bound insulin protease (Duckworth, 1988). Insulin degradation, as previously reviewed (Duckworth *et al.* 1981, Duckworth, 1988), is accomplished through three enzymatic processes: **1.** Glutathione insulin transhydrogenase, a microsomal enzyme that cleaves disulfide bonds and catalyzes not only insulin breakdown but also other disulfide-containing proteins (Varandani, 1972). **2.** Lysosomal enzymes; however, the contribution of this system to insulin metabolism seems very small. **3.** Insulin protease (insulinase), a proteolytic enzyme which is specific for insulin (Brush, 1971) and is predominantly (>90%) located in the soluble fraction of cell homogenates of rat tissues (Kitabchi *et al.* 1972). It accounts for more than 95% of all insulin degrading activity in human muscle (Neal *et al.* 1982), fibroblast (Harris *et al.* 1981), as well as in fat tissues of rats (Kitabchi *et al.* 1972). The current study used the 100,000 g supernatant fractionated from the liver tissues. Since glutathione insulin transhydrogenase is a microsomal enzyme, it is unlikely to be present in the fraction used and its contribution to the observed insulin degrading activity can be ruled out.

In the present study, the TCA precipitation method was used to determine the effect of HCQ on insulin degradation. It has been established that there is a significant correlation (r = 0.96) between the degradation of insulin measured by TCA precipitation and that measured by a double-antibody method (Petersen *et al.* 1982). Hydroxychloroquine significantly reduced insulin metabolism in the cytosolic fraction of livers of both healthy and diabetic animals as indicated by Vm, Km and Vm/Km (Fig. 3-2; Table 3-1). As the Michaelis constant is unaltered by HCQ (Table 3-1), the inhibitory effect of HCQ on insulin metabolism is likely a classic-noncompetitive inhibition.

Interestingly, *in vivo* studies revealed that in diabetic, but not healthy rats sensitivity to insulin was affected by HCQ (Emamibafrani *et al.*, 1995). *In vitro*, however, liver preparation from both healthy and diabetic rats showed reduced insulin metabolism (Table 3-1 and Fig. 3-2). In non-diabetic animals the disturbance in glucose homeostasis induced by HCQ was probably antagonized by a counter-regulatory increase in the concentrations of cortisol, glucagon, growth hormone, catecholamines and/or increase in insulin secretion from β cells. Therefore, in non-diabetic subjects the normal homeostatic mechanisms responsible for insulin-glucose regulation may compensate for possible HCQ-induced perturbation in insulin metabolism and prevent the onset of hypoglycemia. This may explain the discrepancy between our previous *in vivo* and the present *in vitro* observations.

In the present study, it was observed that insulin-deficient animals have decreased insulin degrading activity (Table 3-1; Figs. 3-1B, 3-2). This may be due to a reduction in enzyme synthesis, which may be interpreted as a protective mechanism, such that in the

presence of low levels of insulin, less is degraded. *In vivo* studies, on the other hand, have shown that these diabetic animals have increased insulin clearance caused by an increase in the number of insulin receptors (Sato *et al.* 1991). Therefore, reduced insulin degradation observed *in vivo* in HCQ pretreated STZ-diabetic rats (Chapter 2) might be, at least in part, due to HCQ inhibition of receptor-mediated degradation of insulin as shown to be the case with chloroquine (Bouser *et al.* 1983).

We have shown that the mechanism responsible for the beneficial effect of HCQ on glucose homoeostasis may be partly due to a reduction in intracellular insulin degradation at a postreceptor level. In addition, it is known that chloroquine slows receptor recycling resulting in intracellular insulin accumulation (Smith *et al.* 1989). As an acidotropic agent, HCQ concentrates intracellularly which may inhibit endosomal degradation of the insulin internalized with its receptor. This inference is based on the ability of chloroquine to accumulate in endosomal structures of the cell leading to inactivation of proteolytic enzymes due to an increase in intracellular pH. Furthermore, in lymphocytes, chloroquine appears to enhance binding of insulin to its membrane receptor (Iwamoto *et al.* 1981). It has also been proposed that the dissociation of insulin from its receptor appears to be the rate limiting step in the degradation process of the hormone. Thus, a direct interaction of HCQ with the insulin receptor reduces the rate of dissociation of insulin from its receptor (Smith *et al.* 1989). This condition might increase the biological half-life of the receptor-insulin complex and consequently prolong the action of insulin.

The HCQ concentrations used in this study (2.5-40 mg/mL) represent a considerably high serum level of HCQ. These concentrations, or even higher levels, could

perhaps be achieved intracellularly at the subcellular enzyme compartment. This inference is due to the fact that HCQ and its enantiomers are known to have very high volumes of distribution attributed to extensive tissue uptake (Tett *et al.* 1988, 1990,1993; Ducharme *et al.* 1995). *In vivo* tissue distribution studies have shown that muscle and liver are the two main repositories for HCQ (McChensy, 1983; MacIntyre and Cutler, 1988).

Insulin resistance in patients with NIDDM has been attributed to several factors including pre-receptor and post-receptor defects. It has also been shown that some subjects with insulin resistance exhibit an increased rate of intracellular insulin metabolism (Vasquez *et al.*, 1984; Kitabchi *et al.*, 1972). Hydroxychloroquine may be able to partially reduce the post-receptor increased insulin clearance accelerating insulin-stimulated glucose transport which leads to better management of the disease. Therefore, this drug may have therapeutic potential as an adjuvant in the treatment of both type I and type II diabetic patients. Hydroxychloroquine may be beneficial in insulin-dependent diabetic patients with residual β-cell function, since it sustains higher insulin levels.

In conclusion, the effect of HCQ on insulin degradation appears to be, in part, through inhibition of cytosolic insulin metabolizing enzyme. Thus, HCQ may be able to overcome the post-receptor and intracellular abnormalities such as increased insulin metabolism seen in some type I and II diabetic patients. This may provide a means of diabetic control and better disease management.

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Chapter 4

EFFECT OF EXPERIMENTAL DIABETES MELLITUS AND ADJUVANT ARTHRITIS ON THE PHARMACOKINETICS OF HYDROXYCHLOROQUINE ENANTIOMERS IN RATS*

INTRODUCTION

Hydroxychloroquine (HCQ) is a racemic antimalarial agent which is also an effective disease modifying drug against rheumatoid arthritis (Clark *et al.*, 1993). A well-controlled, double-blind study of HCQ efficacy in patients with rheumatoid arthritis has demonstrated that this drug had a significant benefit on synovitis, pain, and physical disability of recent-onset rheumatoid arthritis (The HERA study group, 1995). Hydroxychloroquine is reported to have lower incidence of retinal damage and gastrointestinal toxicities compared to its analogue chloroquine (Finbloom *et al.*, 1985). A preliminary clinical trial indicated that concomitant HCQ and insulin therapy improved glycemic control by reducing daily insulin requirements by 30% in patients with resistant non-insulin dependent diabetes mellitus (NIDDM) (Quatraro *et al.*,1990). Furthermore, we have recently demonstrated that HCQ reduces metabolic clearance of insulin and consequently improves glycemic control by an HCQ concentration dependent manner in experimental diabetes (Emami *et al.*, 1995). Patients with rheumatic disease may be predisposed towards the development of diabetes mellitus (Martin *et al.*, 1995). Thus, the co-existence of diabetes mellitus and rheumatoid arthritis may increase the risk of

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hypoglycemia where HCQ may sustain higher insulin levels in diabetic patients with some residual β-cell function.

Diabetes mellitus and rheumatoid arthritis are associated with several pathophysiological changes which may potentially alter the pharmacokinetics and pharmacodynamics of drugs. The pathophysiological changes and alterations in glucose homeostasis associated with diabetes mellitus may have fundamental effects on basic cellular processes, resulting in altered handling of many drugs.

Diabetes mellitus has been reported to induce an early increase in glomerular filtration in human (Mogensen, 1971; Madacsy *et al.*, 1976; Osterby and Gundersen, 1975) and animal model of diabetes (Jensen *et al.*, 1981; Harvey *et al.*, 1988), to alter the activity of metabolizing enzymes in humans (Salmela *et al.*, 1980; Zysset and Wietholtz,1988) and animals (Mahachai *et al.*, 1988; Toda *et al.*, 1987; Kato *et al.*, 1970) and to affect the blood concentration of lipoproteins, proteins and free fatty acids (Fraze *et al.*, 1985; Miller *et al.*, 1990; Kosner and Karadi, 1988). Kidney glomerular hyperfiltration has been documented in diabetic patients during the first decade of the disease. Higher glomerular filtration rates (GFR) have also been demonstrated in experimental diabetes. All of these factors may potentially alter the pharmacokinetics and pharmacodynamics of drugs.

Inflammatory conditions such as rheumatic disease are also associated with many pathophysiological changes which may alter drug disposition. Hypoalbuminemia, which is often observed in arthritic subjects, may affect protein binding of drugs (Van Den *et al.*, 1987). A rise in acute phase reacting proteins such as α_1-acid glycoprotein in

inflammatory conditions leads to a decrease in the free fraction for basic drugs (Belpair *et al.*, 1982). Furthermore, acute inflammation decreases hepatic drug metabolism by cytochrome P-450 isoenzymes (Schneider *et al.*, 1981; Ishikawa *et al.*, 1991; Piquette-Miller and Jamali, 1992). These alterations in drug disposition could have clinically significant pharmacodynamic effects.

Adjuvant-induced arthritis and streptozotocin (STZ)-induced diabetes mellitus (STZ-DM) are accepted models for rheumatoid arthritis (Whitehouse, 1988) and diabetes mellitus (Jensen *et al.*, 1981; Harvey *et al.*, 1988) in humans.

Enantioselectivity in the pharmacokinetics of HCQ has been previously studied in rheumatoid arthritic patients receiving chronic racemic (rac) HCQ treatment (McLachlan *et al.*, 1993[a]) and in healthy subjects following a single oral dose of the racemate (Ducharme *et al.*, 1995). Therefore, it is imperative to utilize a stereospecific analytical assay since diseases may not affect the disposition of each enantiomer equally.

To study the effect of diabetes mellitus and adjuvant arthritis on the disposition of HCQ enantiomers, the blood concentrations and urinary excretion of the enantiomers of HCQ were examined in healthy, diabetic, insulin treated diabetic and arthritic rats after a single i.v. administration of racemic HCQ. Blood concentration-time profiles of HCQ enantiomers were also characterized following a single p.o. administration of rac-HCQ to one group of healthy animals. In addition, the *in vitro* binding of HCQ enantiomers to plasma protein and platelets was also determined in arthritic rats and controls using platelet-poor and platelet-rich plasma.

EXPERIMENTAL SECTION

Materials

Streptozotocin (STZ), ortho-toluidine and chloroquine diphosphate (Sigma Chemical Co., St. Louis, MO); Insulin NPH (Iletin I, Eli Lilly and Company, Indianapolis, IN); rac-HCQ sulfate (gift from Sepracor Inc., Marborough, MA); *Mycobacterium butyricum* (Difco Lab., Detroit, MI, USA). All other chemicals and solvents were either reagent or HPLC grade.

Animals

Adult male Sprague-Dawley rats were matched for their initial body weight and housed for at least 3 days before induction of the diseases. Animals had free access to standard laboratory rat chow and water. These animals were assigned to five groups as follows: diabetic (D), insulin treated diabetic (ITD), control (C_D), adjuvant arthritis (A), and control (C_A). A sixth group (PO) consisted of healthy rats who received the drug orally for determination of bioavailability. Bioavailability was estimated by comparing the area under blood concentration-time profiles (AUC) of R- and S-HCQ of these animals with those of C_D rats.

Induction of Diabetes Mellitus

Insulin-dependent diabetes mellitus (IDDM) was induced by a single i.p. injection of 60 mg/kg of STZ. The solution of STZ was prepared immediately before injection by dissolving the powder in 0.1 M citrate buffer (pH 4.2). To confirm the induction of disease, the serum concentration of glucose was measured on day four after injection of STZ and before administration of HCQ. Rats were considered diabetic and included in the

study if their serum glucose was greater than 300 mg/dL. Commencing on the fourth day after induction of diabetes, each rat in the ITD group received a single daily injection of 15 U/kg of insulin s.c. The last insulin injection was performed on day nine after the induction of diabetes (Mehvar, 1991).

Induction of Adjuvant Arthritis

To induce adjuvant arthritis, rats were innoculated intradermally into the tail base with 0.05 mL of heat-killed, freeze-dried *Mycobacterium butyricum* (Difco Lab, Detroit, USA) suspended in squalene (10 mg/mL). Rats were studied approximately 15 days later upon the appearance of arthritis. The degree of arthritis was assessed through visual and objective measures (Piquette-Miller and Jamali, 1992). Hindpaw swelling was quantified by water displacement and measurement of diameter using a caliper. An arthrogram score was obtained by visually grading each hindpaw on a 0 to 4 basis (0, no involvement; 1, involvement of single joint; 2, involvement of > 1 joint and/or ankle; 3, involvement of several joints and ankle with moderate swelling; or 4, involvement of several joints and ankle with severe swelling) and each forepaw on a 0 to 3 basis (0, no involvement; 1, involvement of single joint; 2, involvement of > 1 joint and/or wrist; or 3, involvement of wrist and joints with moderate to severe swelling). A maximum score of 14 could thus be obtained. Animals with an arthrogram score less than 2 were considered non-responders and were excluded from the pharmacokinetic studies. The pharmacokinetics of HCQ were determined on day 15.

Surgery and Animal Maintenance

One day prior to HCQ administration , silastic catheters (0.58 mm i.d. x 0.965 mm o.d.; Clay Adams, Parsippany, NJ) were surgically implanted into the right jugular vein under light methoxyflurane anesthesia (Metofane, Pitman-Moore, Washington Crossing, NJ) and the animals were allowed to recover overnight. Rats had free access to water and were fasted for 16 h prior to drug administration.

Dosing and Sample Collection

Racemic HCQ (40 mg/kg) dissolved in saline solution was administered i.v. to C_D, C_A, D, ITD, and A rats through the jugular vein catheter and p.o. to the PO group. The catheter was then flushed with 0.2 ml of isotonic saline solution. Blood samples (0.2 mL) were collected from the jugular vein cannula before and at 30 min, 1, 2, , 3, 4, 6, 8, 12, 24, and 36 h after p.o. HCQ administration. Following the i.v. dose, two additional samples were also taken at 5 and 10 min post dose. The catheter was then flushed with 0.2 mL of heparin in saline (100 U/mL) following each blood sample collection. All samples were stored at -20°C until analyzed. Total urine output was collected up to 72 h and, after recording the volume, an aliquot was stored and frozen for later analysis.

Binding to Plasma Protein and Platelets

The *in vitro* binding of HCQ enantiomers to protein and platelets was determined by an ultrafiltration technique using platelet-poor plasma and platelet-rich plasma from healthy and arthritic rats. For the collection of platelet-rich plasma, the blood was anticoagulated with (Na_2) EDTA (Vacutainer tubes, Becton Dickinson, Rutherford, NJ) and centrifuged at 150 *g* for 10 min. The top layer, containing the platelet rich plasma,

was collected. Platelet-poor plasma was obtained after centrifugation of whole blood at 1800 *g* for 10 min. Pooled platelet-poor plasma and platelet-rich plasma (3 mL) were spiked with racemic HCQ at concentrations of 0.5, 1, 2, 5, and10 μg/mL. Prior to ultrafiltration, the pH of each HCQ spiked solution was measured and, if necessary, adjusted to pH 7.4 ± 0.1 using a small volume (<20 μL) of 1 N HCL. The ultrafiltration of 1 mL aliquots of each sample placed in the reservoir of each ultrafiltration unit was accomplished at 1850 *g* for 45 min using Diaflo membranes attached to Amicon ultrafiltration tubes (Micon Micropartition System, Amicon Div., W. R. Grace and Co. Danvers, MA, USA). This was done after the ultrafiltration units containing the samples were allowed to incubate at 37°C for 1 h. The reservoir of each unit was capped to prevent sample evaporation and pH changes during the ultrafiltration procedure. The percent unbound of each HCQ enantiomer, F_u, was determined using the expression:

$$\%F_u = (C_{uf} / C_t) \cdot 100\%$$

where C_t was the total (bound and unbound) concentration of individual enantiomer in each sample before ultrafiltration, and C_{uf} was the ultrafiltrate HCQ concentration of each enantiomer.

As non-specific binding of drug to the ultrafiltration unit or membrane can result in overestimation of plasma protein binding, the extent of such binding of HCQ enantiomers was determined in phosphate buffer at physiologic pH and temperature. Total HCQ enantiomer binding to both the filter unit and membrane was negligible and non-stereoselective.

Measurement of Serum Glucose

Serum glucose concentrations were determined using the ortho-toluidine method (Linne *et al.*, 1979). To 50 μL of serum was added 3 mL of ortho-toluidine reagent and the mixture incubated for 12 min in a boiling water bath. Samples were then cooled in an ice bath for 5 min and left at room temperature for another10 min. The color intensity was read at 630 nm. The color was found to be stable for at least one hour.

Assay of HCQ Enantiomers

Concentrations of HCQ enantiomers in blood and urine were measured using a previously described HPLC method (Brocks *et al.*, 1992). Briefly, after addition of chloroquine (internal standard), samples (0.2 mL) were alkalinized and extracted with 5 mL of diethyl ether. After solvent evaporation the residues were derivatized with a solution of (+)-di-O-acetyl-L-tartaric anhydride at 45°C for 30 min. The resulting diastereomers were then resolved using a C8 analytical column with a mobile phase consisting of 0.05 M KH_2PO_4 (pH 3):methanol:ethanol:triethylamine (80:20:1:0.08). The UV detection wavelength was set at 343 nm.

Pharmacokinetic Data Analysis

The data were fitted to a biexponential equation using the computer program PCNONLIN 4.1 (SCI Software, Lexington, Kentucky). All estimates maintained a minimum correlational coefficient of 0.97. The area under the first-moment-time curve ($AUC_{0-\infty}$) was calculated by the equation $A/\alpha + B/\beta$, where A and B are intercept coefficients. The apparent distribution and elimination half-lives ($t_{1/2}$) were calculated as

$0.693/\alpha$ and $0.693/\beta$, respectively. The systemic clearance (CL) was calculated from Dose/$AUC_{0-\infty}$, and the mean residence time from $AUMC_{0-\infty}$ /$AUC_{0-\infty}$, the volume of distribution at steady state (V_{ss}) from CL•MRT.

Renal clearance (Cl_r) was determined as the slope of the cumulative urinary recovery of each HCQ enantiomer during the time interval from 0 to t (X_{U0-t}) *vs* the corresponding area under the blood concentration-time curve value (AUC_{0-t}). Non-renal clearance (CL_{nr}) was estimated for each rat by subtraction of CL_r from CL.

Statistical Analysis

Pharmacokinetic differences between A and C_A rats and differences between pharmacokinetic parameters of each individual enantiomers of HCQ were tested using unpaired and paired Student's two-sided t-test, respectively. The differences among the three groups of C_D, D, and ITD rats were determined based on a single factor analysis of variance (ANOVA). Further analysis of the means was achieved using Duncan's multiple range test. Differences were considered significant at $P < 0.05$. Data are presented as mean ± S.D.

RESULTS

Induction of Diseases

Serum glucose levels of C_D, D, and ITD rats and mean total body weights of all animals studied are presented in table 4-1. Prior to induction of diseases the body weights of C_D rats did not differ from those of D rats. Similarly, the body weights of C_A rats were not different from those of A rats. However, 10 days after induction of diabetes, at the time of the pharmacokinetic studies, the body weights of D rats were slightly but

significantly lower compared to the respective controls. Serum glucose concentrations of diabetic rats were significantly higher than those of C_D and ITD rats. Daily injection of 15 U/kg of insulin, before commencement of the pharmacokinetic studies, resulted in normal serum glucose concentrations in the ITD animals. No difference was observed for body weight after induction of arthritis in A rats. In these animals, skin nodules on the ears and tail and swelling of the hind and fore paws were observed (arthrogram score, 7.6± 4.1).

Table 4-1. Body weights and serum glucose levels of different groups of rats.

	Controls		Test groups		
	C_D	C_A	D	ITD	A
Body weight (g)					
Before induction of disease	260 ± 20	263 ± 25	274 ± 31	258 ± 21	258 ± 18
At the time of PK studies	343 ± 36	403 ± 30	310 ± 33[a]	360 ± 15	386 ± 28
Serum glucose (mg/dL)	139 ± 5	NA[c]	414 ± 49[b]	116 ± 34	NA[c]

[a] Significantly different from C_D rats ($P < 0.05$)
[b] Significantly different from C_D and ITD rats
[c] Not applicable

Pharmacokinetics of HCQ Enantiomers in Healthy Rats

Blood concentration-time courses of the HCQ enantiomers following p.o. and i.v. administration of rac-HCQ in healthy rats are shown in figures 4-1 and 4-2. Pharmacokinetic parameters are presented in tables 4-2 and 4-3. The disposition of HCQ in blood was stereoselective, with the CL of S-HCQ being significantly higher than R-HCQ. Consequently, the $AUC_{0-\infty}$ of R-HCQ was significantly higher as compared with that of S-HCQ. Half-lives of the two HCQ enantiomers, obtained from the linear portion of the blood concentration-time course (range 5 to 8.5 h), were not different. A

significantly higher apparent volume of distribution was observed for S-HCQ as compared with R-HCQ after both i.v. and p.o. administration. The CL_r of S-HCQ was significantly higher than that of R-HCQ. There was , however, a trend towards higher CL_{nr} for S-HCQ as compared with the R enantiomer. Urinary excretion of the unchanged HCQ enantiomers was stereoselective, with S-HCQ being present in greater amounts. Following oral dosing the difference in C_{max} between enantiomers was statistically significant (1.13 ± 0.37 and 0.94 ± 0.32 mg/L for R and S-HCQ respectively), but there was no difference in the T_{max} values of the enantiomers (2.63 ± 1.1 and 2.00 ± 0.71 h, for R and S-HCQ respectively). The $AUC_{0-\infty}$ of R-HCQ (9.21 ± 1.33 (mg/L)h) was significantly higher as compared with that of S-HCQ (7.39 ± 1.35 (mg/L)h , $P < 0.003$). The bioavailability of R-HCQ was not different from that of the S enantiomer (0.79 ± 0.13 and 0.72 ± 0.15 for R- and S-HCQ respectively).

Effect of Diabetes Mellitus on Pharmacokinetics of HCQ Enantiomers

Diabetes resulted in a significant increase in CL of both enantiomers of HCQ (Fig. 4-2, Tables 4-2 and 4-3), the effect being more pronounced for S-HCQ (80% and 100% for R- and S-HCQ respectively). The increase in CL apparently resulted from a significant increase in CL_r (70% and 62.5% for R- and S-HCQ respectively) and CL_{nr} (100% and 145% for R- and S-HCQ respectively) values for the enantiomers. Higher CL of the enantiomers in diabetic rats was reflected in significantly lower blood concentrations and AUCs for both enantiomers in this group of rats. The decrease observed in V_{ss} of D rats (13% and 25% for R-HCQ and S-HCQ respectively) was not significant compared to C_D rats. Therefore, the diabetes-induced increase in CL was responsible for the significant

reduction in the half-lives of R- and S-HCQ (51% and 54% for R- and S-HCQ respectively) when compared to those of respective controls. Although the Cl_r of HCQ enantiomers were significantly increased in D rats as compared with healthy rats, the contribution of renal excretion to overall elimination of HCQ enantiomers in D (26.7%) and C_D (28.8%) were not different. This was due to higher Cl_{nr} values in D rats. Insulin treatment reversed diabetes-induced alterations in the pharmacokinetics of HCQ enantiomers (Tables 4-2 and 4-3). None of the pharmacokinetic parameters of HCQ enantiomers in ITD were statistically different from those in C_D rats.

Effect of Adjuvant Arthritis on the Pharmacokinetics of HCQ Enantiomers

The pharmacokinetics of HCQ were significantly affected by adjuvant-induced arthritis (Fig. 4-3, Tables 4-2 and 4-3). The CL values for both enantiomers in A rats were significantly lower as compared to those of C_A rats (61% and 50% for R- and S-HCQ, respectively), resulting in significantly increased AUCs. The mean $AUC_{0-\infty}$ of R-HCQ was significantly higher than S-HCQ in both A and C_A rats (Tables 4-2 and 4-3). R:S AUC ratios in A rats did not differ significantly from C_A rats. Disease severity (arthritic index) correlated strongly with $AUC_{0-\infty}$ for both HCQ enantiomers ($r = 0.95$ and 0.96 for R and S-HCQ, respectively, Fig. 4-4).

Binding to Plasma Protein and Platelets

Binding of HCQ enantiomers to platelets and plasma proteins was not concentration dependent within the examined concentration range (Fig. 4-5A). The mean unbound fraction (F_u) of HCQ in platelet poor plasma (Fig. 4-5B) showed stereoselectivity, with R-HCQ being more unbound than S-HCQ (0.62 ± 0.034 and $0.50 \pm$

0.065 for R- and S-HCQ respectively). The mean F_u of S-HCQ, however, was significantly greater than that of R-HCQ when platelet rich plasma was used (0.26 ±0.015 and 0.32 ± 0.015 for R- and S-HCQ respectively). The F_u of the R-HCQ and S-HCQ were 49.4% and 50.5% lower in platelet rich plasma of arthritic rats as compared to that of normal rats (Fig. 4-5B). The correlation between the number of platelets in plasma and the F_u is illustrated in figure 4-5C. Strong correlation was observed between the number of platelets in plasma and F_u for both R ($r = 0.85$) and S ($r = 0.81$) enantiomers of HCQ.

Table 4-2. Pharmacokinetic parameters of R-HCQ in controls, diabetic, insulin treated and arthritic rats.

INDICES	Controls		Disease states			Statistical analysis			
	C_D	C_A	D	ITD	A	C_D *vs* D	C_D *vs* ITD	D *vs* ITD	C_A *vs* A
$AUC_{0-\infty}$ (mg/L)*h	12.1±2.31*	12.8±1.85*	6.8±1.47*	11.6±1.47	19.5±3.12*	+	-	+	+
CL_{TB} (L/h/kg)	1.69±0.32*	1.61±0.25*	3.04±0.69*	1.74±0.22	1.05±0.15*	+	-	+	+
CL_r (L/h/kg)	0.61±0.14*	ND	1.04±0.13	0.72±0.17	ND	+	-	+	ND
CL_{nr} (L/h/kg)	1.00±0.15*	ND	2.00±0.57*	1.02±0.16	ND	+	-	+	ND
Xu_{0-109} (% dose)	28.8±0.74	ND	26.7±3.29	27.4±1.52	ND	-	-	-	ND
$\beta t_{1/2}$ (h)	6.95±1.30	7.12±1.23	3.40±1.24	5.64±0.93	7.91±1.99	+	-	+	-
V_{ss} (L/kg)	14.6±2.74*	14.4±3.34*	12.6±2.47	13.0±2.61	10.6±2.09*	-	-	-	+

Table 4-3. Pharmacokinetic parameters of S-HCQ in controls, diabetic, insulin treated and arthritic rats.

INDICES	Controls		Disease states			Statistical analysis			
	C_D	C_A	D	ITD	A	C_D *vs* D	C_D *vs* ITD	D *vs* ITD	C_A *vs* A
$AUC_{0-\infty}$ (mg/L)*h	10.6±1.93*	10.5±2.05*	5.42±1.43*	10.2±1.77	15.8±2.18*	+	-	+	+
CL_{TB} (L/h/kg)	1.93±0.34*	1.96±0.33*	3.90±1.08*	2.01±0.33	1.30±0.19*	+	-	+	+
CL_r (L/h/kg)	0.80±0.21*	ND	1.30±0.28	0.96±0.24	ND	+	-	+	ND
CL_{nr} (L/h/kg)	1.06±0.13*	ND	2.60±0.85*	1.05±0.16	ND	+	-	+	ND
Xu_{0-109} (% dose)	34.3±1.17	ND	31.0±4.38	32.5±2.38	ND	-	-	-	ND
$\beta t_{1/2}$ (h)	7.5±2.2	7.01±1.03	3.4±1.24	5.40±1.02	8.66±2.80	+	-	+	-
V_{ss} (L/kg)	17.2±4.04*	16.5±3.47*	12.8±3.93	13.9±3.17	13.6±3.74*	-	-	-	+

* Significantly different from other enantiomer ($P < 0.05$). (+) Significantly different ($P < 0.05$). (-) Not different ($P > 0.05$). (ND) Not determined.

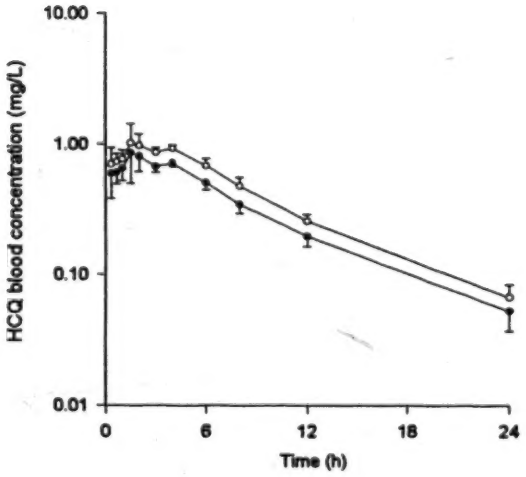

Figure 4-1. Blood concentration-time profiles of HCQ enantiomers in rats following oral administration of racemic HCQ. R-HCQ (O); S-HCQ (●). (n = 5), Values are means with S.D. bars.

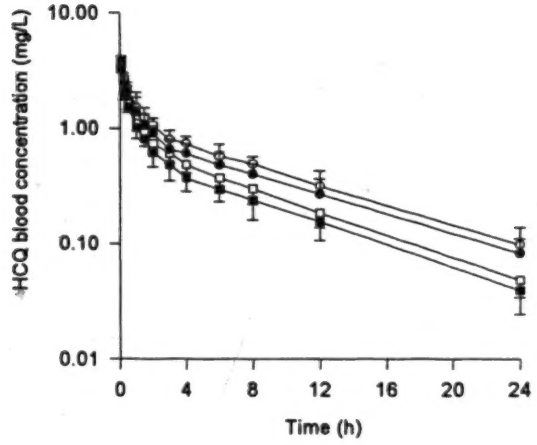

Figure 4-2. Blood concentration-time profiles of HCQ enantiomers in C_D and D rats following adminstration of racemic HCQ. C_D: R-HCQ (O); S-HCQ (●), D: R-HCQ (□); S-HCQ (■). (n =5), Values are means with S.D. bars.

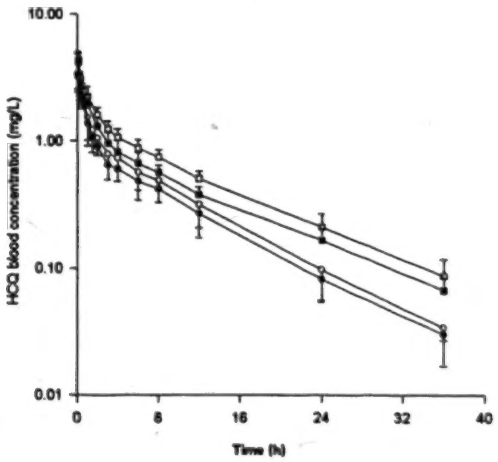

Figure 4-3. Blood concentration-time profiles of HCQ enantiomers in C_A and A rats following administration of racemic HCQ. C_A: R-HCQ (O); S-HCQ (●), A: R-HCQ (□); S-HCQ (■). (n = 5), Values are means with S.D. bars.

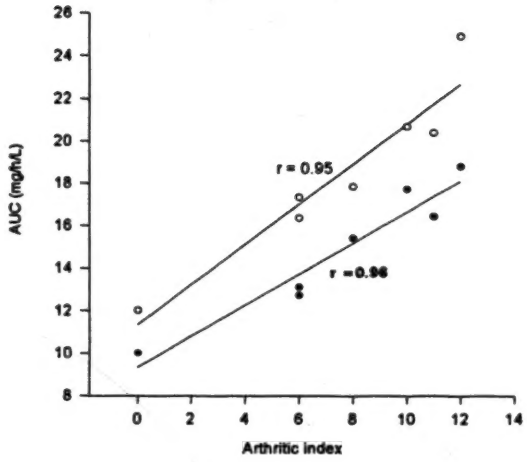

Figure 4-4. Area under the concentration-time course of HCQ enantiomers vs athritic index in arthritic rats. R-HCQ (O); S-HCQ (●).

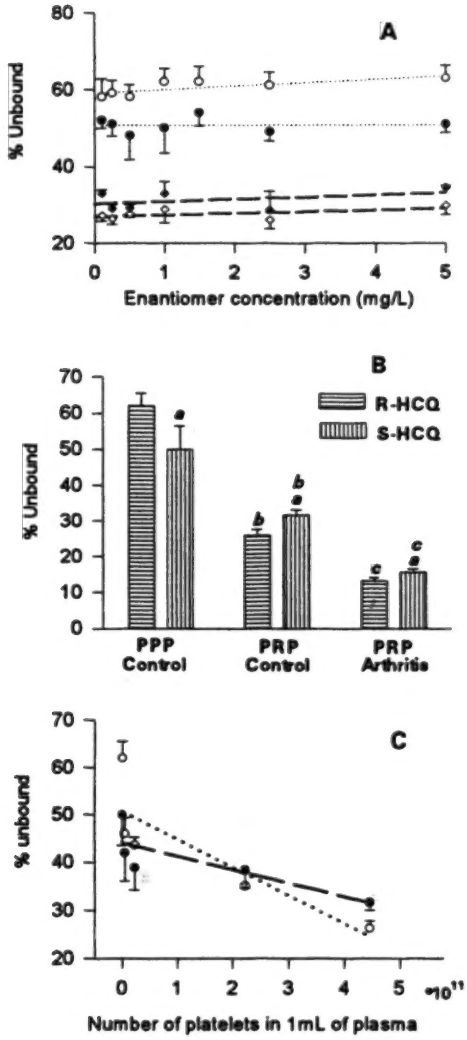

Figure 4-5. **(A)** Mean percent unbound *vs* concentration of HCQ enantiomers in: Platelet-poor plasma (PPP), R-HCQ (O---O); S-HCQ (●---●); Platelet-rich plasma (PRP), R-HCQ (O—O); S-HCQ (●—●). (B) Mean percent unbound of HCQ enantiomers in platelet poor plasma and platelet rich plasma of healthy and arthritic rats. (C) Mean percent unbound of HCQ enantiomers as a function of platelet numbers in rat plasma. R-HCQ (O); S-HCQ (●). All samples were analyzed in triplicate. Values are mean ± S.D.

a, Significantly different from R-HCQ; *b*, Significantly different from platelet-poor plasma of controls; *c*, Significantly different from platelet-rich plasma of controls.

DISCUSSION

To estimate the pharmacokinetic parameters of HCQ enantiomers, blood concentration-time data following i.v. administration were best fitted to a two-compartment open model. In humans, however, the pharmacokinetics of HCQ and its enantiomers follows a three compartment model (Ducharme *et al.*, 1995). Since HCQ blood levels beyond 24-36 h after dosing were below the sensitivity of the HPLC assay, we were unable to characterize more than a two compartment model using blood data. A comparison of the blood concentration-time profile *vs* urinary excretion rate-time curve, however, suggests that elimination of HCQ enantiomers in the rat follows a three compartment model. When the $AUC_{t-\infty}$ was estimated using the last blood concentration and elimination rate constant obtained from the urinary excretion rate-time plot, it was noticed that the contribution of the $AUC_{t-\infty}$ to the overall $AUC_{0-\infty}$ was about 10%. The volume of distribution of HCQ enantiomers was found to be 25 times greater than rat total body water (0.7 L/kg). This is qualitatively parallel to that observed in human studies (Ducharme *et al.*, 1995). The large volume of distribution of weak bases such as HCQ is attributed, at least in part,to tissue uptake by ion trapping in lysosomes.

As observed after single (Ducharme *et al.*, 1995) and multiple (McLachlan *et al.*, 1993[a]) doses of rac-HCQ to humans, higher blood concentrations of R-HCQ compared with S-HCQ were seen in the rat model. The mean R:S AUC ratio after p.o. administration of the drug observed in rats was similar to that observed in humans (1.3 *vs* 1.8 respectively). The higher R-HCQ concentrations confirm the operation of a

stereoselective process(s) in the disposition of HCQ enantiomers. In the present study, it is shown that the mean absorption half-lives, T_{max} values and bioavailability of HCQ enantiomers were not different, while C_{max} values for R-HCQ were found to be significantly higher compared to S-HCQ ($P < 0.004$). This observation parallels reports that the T_{max}, absorption half-lives and the fraction of the dose absorbed were similar for both enantiomers in humans (Mclachlan *et al.*, 1994). This finding is also consistent with the observation of Ducharme (Ducharme *et al.*, 1995). Therefore, it appears that the stereoselective disposition of HCQ is due to enantioselective clearance.

An *in vitro* study revealed that the binding of HCQ to human plasma protein is enantioselective in favor of S-HCQ (Mclachlan *et al.*, 1993[b]). In the current study, the plasma protein binding of HCQ was also found to be stereoselective. The R- and S-HCQ were 62% and 50% unbound respectively.

In the present study, CL_r of both HCQ enantiomers were found to be approximately 2.5 times greater than the GFR in healthy rats which indicates that tubular secretion has an important contribution to the overall renal elimination of this drug. Therefore, the differences in renal clearance of the enantiomers may, in part, result from stereoselectivity in the process of active secretion. These findings parallel those found in humans (Ducharme *et al.*, 1995). In light of this similarity with humans, the rat appears to be a good model for further pharmacokinetic studies of HCQ enantiomers.

Many diabetic patients develop serious complications during the course of the disease resulting in consumption of more medications as compared with nondiabetics. Furthermore, the pathophysiological changes and alterations in glucose homeostasis

associated with diabetes mellitus may influence the basic cellular processes thereby altering the pharmacokinetics and pharmacodynamics of drugs. An elevated GFR in the early phase of IDDM has been reported in human (Mogensen, 1971; Madacsy *et al.*, 1976; Osterby and Gundersen, 1975) and animal models of diabetes (Jensen *et al.*, 1981; Harvey *et al.*, 1988). In diabetic patients the volumes of the individual capillary lumina and of the individual glomerular cells were enlarged possibly due to either a pressure-induced unfolding of the capillary wall or an increase of the capillary wall area (Osterby and Gundersen, 1975). These pathophysiological changes result in kidney hyperfiltration. Hyperfiltration is especially more pronounced in newly diagnosed patients; i.e. acute onset of disease or within 3-6 weeks of treatment with insulin (Mogensen, 1971). The elevation of glomerular filtration rate persists during the first decade despite insulin therapy and adequate metabolic control (Osterby and Gundersen, 1975: Mogensen, 1971). Subsequently, GFR significantly decreases in patients with long-standing IDDM (Mogensen, 1971).

Despite ample evidence regarding alteration in renal function, the influence of diabetic complications on drug disposition has received relatively little attention. The effect of diabetes on the disposition of drugs has been examined in diabetic children who have had the disease for less than five years. For instance, the glomerular filtration rate is significantly higher and the carbenicillin $t_{1/2}$ significantly shorter when compared to healthy children (Madacsy *et al.*, 1976).

The data obtained in the present study indicates that STZ-induced diabetes mellitus resulted in a significant increase in both renal and non-renal elimination of HCQ

enantiomers. The increased CL_r is consistent with a significantly higher kidney GFR reported in STZ-induced diabetic rats compared to controls (Jensen *et al.*, 1981). Renal clearance of HCQ enantiomers (10.3 and 13.3 mL/min/Kg for R- and S-HCQ respectively) in healthy rats was found to be 2.3 (for R) and 3 times (for S) greater than GFR (4.5 mL/min/kg). Creatinine clearance in STZ-induced diabetic rats (9 mL/min/kg), on average, has been reported to be 4.5 mL/min/kg higher than respective values in healthy animals (Mehvar, 1991). The CL_r value of R- and S-HCQ in STZ-induced diabetic rats, however, increased by 7.1 and 8.4 mL/min/kg, respectively. This suggests that diabetes-induced glomerular hyperfiltration is apparently associated with a parallel increase in tubular secretion of HCQ enantiomers in diabetic rats.

Hepatic drug metabolism, mediated by cytochrome P-450 enzymes, is altered in certain disease states, including diabetes mellitus, in both humans (Salmela *et al.*, 1980; Zysset and Wietholtz,1988) and diabetic animals (Mahachai *et al.*, 1988; Toda *et al.*, 1987; Kato *et al.*, 1970). The direction and extent of the diabetic alteration in metabolism, however, are dependent on several factors such as acuteness and type of the disease, duration of diabetes, gender, species, and the substrate under study. For instance, elimination of antipyrine, a marker for hepatic intrinsic clearance, was found to be faster in patients with treated IDDM (Zysset and Wietholtz,1988) and also in non-insulin dependent diabetic patients with normal liver function (Salmela *et al.*, 1980). A similar effect was found in STZ-induced diabetic rats (Mahachai *et al.*, 1988). Metabolism of aminopyrine, on the other hand, was shown to be slower in both alloxan- and STZ-induced diabetic male rats (Toda *et al.*, 1987) but faster in female rats (Kato *et al.*, 1970).

It seems that uncontrolled diabetes causes an overall increase in cytochrome P-450 in the rat, while the activity of isozymes under androgen control decreases (Toda, 1987; Favreau and Schenkman, 1988).

Our results indicate that STZ-induced diabetes mellitus causes a significant increase in CL_{nr} of both HCQ enantiomers (Tables 4-2, 4-3). However, the extent of increase was more pronounced for S-HCQ than R-HCQ. The total drug excreted in urine indicates that about 70% of the administered dose is eliminated through non-renal pathways, presumably via hepatic metabolism.

Blood concentrations of lipoproteins and proteins are known to be different in diabetic rats compared to controls (Miller *et al.*, 1990; Kosner and Karadi, 1988). It is also well known that free fatty acids are elevated in the plasma of patients with either IDDM or NIDDM (Fraze *et al.*, 1985). Elevated free fatty acids, and glycosylation of plasma protein in diabetes mellitus may affect the extent of binding of basic and acidic drugs (Gwilt *et al.*, 1991). In addition, extra- and intra-cellular volumes are elevated in experimental diabetes mellitus (Harvey *et al.*, 1988). Hence, the volume of distribution of drug may be altered in diabetic states. Our results indicate that HCQ does not bind to plasma proteins to any significant extent. An alteration in plasma protein binding, therefore, is not expected to have a significant impact on the pharmacokinetics of this drug. Increased binding to free fatty acids whose levels increase in diabetes mellitus, may also occur. Any change in free fatty acid binding may have been masked by elevation in the volume of extra- and intra-cellular fluids. This may be an explanation for the insignificant changes in volume of distribution we have observed.

As compared to control, the elimination rate constants of the HCQ enantiomers significantly increased in diabetic rats leading to a significant decrease in elimination $t_{1/2}$ of the enantiomers. This may stem from the fact that an increase in the systemic clearance of the enantiomers was not associated with an increase in volume of distribution.

Inflammatory conditions are associated with altered plasma protein concentrations. Hypoalbuminemia, which is often observed in arthritic subjects, may affect protein binding of acidic drugs. In patients with rheumatoid arthritis, significantly increased free fractions of naproxen, due to an arthritis-induced reduction in plasma albumin concentrations, resulted in significant increases in the volume of distribution and the apparent clearance of naproxen (Van Den *et al.*, 1987). Plasma concentrations of the acute phase protein, α_1-acid glycoprotein, are also greatly increased in inflammatory conditions (Belpaire *et al.*, 1982). Since basic drugs have an affinity for this protein their binding is often increased in diseases such as arthritis.

The impairment of drug metabolism in experimental models of inflammation has been well established. Following induction of adjuvant arthritis in rats, liver microsomal oxidative enzyme activity and cytochrome P-450 content were reduced (Ishikawa *et al.*, 1991). Propranolol levels have been reported to be significantly higher than normal in patients with inflammatory diseases (Schneider *et al.*, 1981) as well as in rats afflicted with inflammation (Piquette-Miller and Jamali, 1992). These increases have been explained by both decreased metabolism and increased protein binding.

Hydroxychloroquine, a weak base which moderately binds to plasma proteins, has a low hepatic extraction ratio from blood in humans (Tett *et al.*,1988) and rats (Tables 4-

2, 4-3). In man and rats, its elimination is mainly dependent on biotransformation in the liver (Tett *et al.*,1988) generating three metabolites, desethylchloroquine, desethyhydroxychloroquine and bisdesethylchloroquine (MacChesney, 1983). Decreased distribution as a result of an elevation in α_1-acid glycoprotein, increase in the number of platelets, and reduction in the liver's intrinsic ability to metabolize the drug, may result in higher HCQ blood concentrations. It has been shown that the number of platelets, one of the major HCQ uptake blood fractions (Brocks *et al.*, 1994), rises in active rheumatoid arthritis and correlates with both clinical and laboratory parameters (Farr *et al.*, 1983).

Inflammation in adjuvant-induced arthritic rats resulted in significant decreases in CL and V_{ss} for both HCQ enantiomers. The decreased volume of distribution in arthritic rats indicates that lowered CL could, in part, be due to an overall change in protein and platelet binding. This could be supported further by a significant decrease in the free fraction of HCQ enantiomers in platelet-rich plasma of arthritic rats as compared to that of healthy animals. However, the reduction in V_{ss} (21% and 26% for R- and S-HCQ respectively) cannot solely account for decreased CL (approximately 35% for both HCQ enantiomers). Thus, in addition to increased binding of the drug, a suppressed intrinsic clearance is likely to play a role in the increased blood concentrations. The observed reduction in CL of HCQ enantiomers could also be due to an alteration in the liver intrinsic ability to metabolize the drug. The low hepatic extraction ratio of HCQ enantiomers excludes the possibility that changes in the hepatic blood flow could be responsible for the decrease in CL of HCQ enantiomers. Furthermore, it has been shown that hepatic blood flow remains unchanged in inflammatory states (Walker *et al.*, 1986).

As the clearance of HCQ was found to be decreased in adjuvant-induced arthritis, we subsequently examined the influence of inflammation severity on its disposition. The progressively elevated AUCs of HCQ enantiomers with increased inflammation indicate that a strong relationship exists between disease severity and reduced HCQ clearance. It is likely that a similar relationship between the magnitude of inflammation and altered HCQ disposition exists in humans.

In conclusion, animal models of diabetes mellitus and adjuvant arthritis were used to study the effects of these diseases on the pharmacokinetics of HCQ enantiomers. Our study showed that rat seems to be an appropriate animal model in which to study the pharmacokinetics of HCQ enantiomers. Experimentally induced diabetes significantly increased the systemic clearance of both enantiomers of HCQ by increasing renal and non-renal clearance. Adjuvant-induced arthritis, on the other hand, caused a significant decrease in systemic clearance of HCQ enantiomers through increased binding and a decreased intrinsic clearance. The effect of the diseases, however, was not stereoselective as both enantiomers were affected almost equally. Further studies in diabetic and arthritic patients are needed to evaluate the relevance of these findings for humans.

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Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

This study investigated the potential hypoglycemic action of hydroxychloroquine (HCQ) and its impact on insulin and glucose homeostasis in healthy and diabetic rats. The inhibitory effect of this drug on insulin metabolism was also studied in cytosolic fractions of liver homogenates in both healthy and diabetic rats. The effect of experimental diabetes mellitus and adjuvant-induced arthritis on the disposition of HCQ enantiomers was also investigated.

In healthy rats, HCQ does not alter fasting serum glucose concentrations, insulin sensitivity, or glucose tolerance. This suggests that an impairment of glucose homeostasis is unlikely to result from HCQ treatment. It is possible that in healthy animals the normal homeostatic mechanisms responsible for insulin-glucose regulation, including decreased insulin concentrations and/or a counter-regulatory increase in the levels of cortisol, glucagon, growth hormone, and cathecholamines, may compensate for possible HCQ-altered insulin metabolism. *In vitro* studies using cytosolic fraction of healthy rat liver homogenates containing an insulin metabolizing enzyme (insulinase), showed a significant reduction in insulin metabolism. Thus a discrepancy between *in vitro* and *in vivo* studies exists. This may stem from the fact that under *in vitro* conditions, the counter-regulatory mechanism explained above is not present. Unlike quinine, which may cause profound hypoglycemia *in vivo,* presumably via release of insulin from pancreatic β-cells (Philips *et al.,* 1986), HCQ and chloroquine (Smith *et al.,* 1987) have no appreciable effect on plasma glucose and insulin concentrations in healthy states. There seems to be no

clinically significant effect in rheumatic patients with normal glucose tolerance receiving therapeutic doses of HCQ. To the best of our knowledge, there are no clinical reports to indicate increased plasma insulin and/or reduced plasma glucose levels in arthritic patients taking HCQ as a disease-modifying agent.

In diabetic rats treated with HCQ for ten days, following an i.v. insulin challenge a significant decrease in the area under percent reduction in glucose time-profiles and a significant increase in the area under percent increase insulin-time profiles were observed. A high degree of correlation was found between the area under percent glucose reduction-time profile with HCQ blood concentrations and dose. The correlation of area under percent increased insulin-time course with HCQ blood concentrations also showed a significant linear relationship. However, such linearity seemed to be less noticeable than that of area under percent glucose reduction-time profile and HCQ blood levels perhaps due to the smaller number of data points used to perform this correlation. Correlations for all respective measured effects seemed to be better with HCQ blood concentrations rather than dose data. Insulin resistance, a common feature of diabetes mellitus, has been attributed to several factors including pre-receptor and post-receptor defects. A postbinding defect in insulin action must be responsible for the observed insulin resistance in patients with well-established hyperglycemia (Arner *et al.*, 1986). Interestingly, it has been shown that some subjects with insulin resistance exhibit an increased rate of intracellular insulin degradation (Vasquez *et al.*, 1985). This may imply that HCQ is partially able to overcome the post-receptor abnormality responsible for insulin resistance by increasing postreceptor insulin concentrations which in turn accelerate the insulin-

stimulated glucose transport. This may provide a means of diabetes control and better disease management. Hydroxychloroquine may sustain higher insulin levels and, therefore, has therapeutic potential in the treatment of patients with residual β-cell function. Clinically, improved glycemic control may decrease diabetic-induced co-morbidity and improve quality of life

Hydroxychloroquine may exert its beneficial effect in diabetes by several mechanisms. Theoretically, HCQ should behave in a manner similar to chloroquine. Chloroquine, which is structurally very similar to HCQ, has been used to study intracellular insulin processing. *In vitro* studies suggest that chloroquine reduces intracellular insulin degradation, increases intracellular insulin accumulation, slows receptor recycling, and stimulates insulin-mediated glucose transport (Smith *et al.*, 1989; Cynober *et al.*, 1987). As an acidotrophic agent, chloroquine concentrates in endosomes which may result in inhibition of endosomal degradation of insulin internalized with its receptor (Varandani *et al.*, 1982; Smith *et al.*, 1989). The drug also may enhance binding of insulin to its membrane receptor (Iwamoto *et al.*, 1981). The insulin-sparing effect of HCQ observed in HCQ-treated diabetic rats was due to reduction in insulin metabolism as indicated by greater insulin levels observed following i.v. administration of insulin in diabetic rats. Improvement in glucose control observed in these animals may have been mediated by the effect of insulin on cellular glucose transport. Our *in vitro* studies of the inhibition of insulin metabolism by HCQ revealed a significant reduction in insulin metabolism in the cytosolic fraction of liver homogenates of healthy and diabetic rats. Our *in vitro* data indicates that a direct interaction of HCQ with the insulin metabolizing

enzyme present in the cytosolic fraction of liver cells could be involved in inhibition of insulin metabolism. Since experimental evidence demonstrates that insulin is metabolized in the same manner in muscle and adipose tissues, it is conceivable that this drug may inhibit insulin degradation in other tissues as well. It was also observed that insulin-deficient animals have decreased insulin degrading activity. This may be due to a reduction in enzyme synthesis, which could be interpreted as a protective mechanism, such that less is degraded in the presence of low levels of insulin. *In vivo* studies, on the other hand, have shown that these diabetic animals have increased insulin clearance caused by an increase in the number of insulin receptors (Sato *et al.* 1991). Therefore, reduced insulin degradation in our HCQ pretreated diabetic rats might be, at least in part, due to HCQ inhibition of receptor-mediated degradation of insulin as shown to be the case with chloroquine (Bouser *et al.* 1983).

From the results obtained in the pharmacokinetic studies of HCQ enantiomers, it appears that the rat is an appropriate animal model in which to study the pharmacokinetics of HCQ. In both humans and rats there is a slight but significant stereoselective disposition of the drug (Ducharme *et al.*, 1995). Similarities exist between the two species in that the renal clearance of S-HCQ was greater than that of the R enantiomer (Ducharme *et al.*, 1995). Blood concentration-time data following i.v. administration of racemic HCQ were best fitted to a two-compartment open model. In humans, however, the pharmacokinetics of HCQ and its enantiomers follows a three compartment model (Ducharme *et al.*, 1995; Tett *et al.*, 1988). A comparison of the blood concentration-time profile *vs* urinary excretion rate-time curve, however, suggests that elimination of HCQ

enantiomers in the rat follows a three compartment model. The volume of distribution of HCQ enantiomers was found to be 25 times greater than rat total body water (0.7 L/kg). This is qualitatively parallel to that observed in human studies (Ducharme *et al.*, 1995). As observed in humans, higher blood concentrations of R-HCQ compared with S-HCQ were seen in the rat model. The mean R:S AUC ratio after p.o. administration of the drug observed in rats was similar to that observed in humans (1.3 *vs* 1.8 respectively). It was shown that the mean absorption half-lives and the fraction of the dose absorbed were similar for both enantiomers, as is observed in humans (McLachlan *et al.*, 1994). Similar to humans, the plasma protein binding of HCQ was found to be stereoselective in favour of the S enantiomer (McLachlan *et al.*, 1993).

Diabetes caused a significant change in the elimination of HCQ whereas distribution was mainly unaffected. Diabetes mellitus resulted in a significant increase in both renal and non-renal elimination of HCQ enantiomers. The increased renal clearance is consistent with a significantly higher kidney GFR reported in streptozotocin-induced diabetic rats (Jensen *et al.*, 1981). It was found that diabetes-induced glomerular hyperfiltration is apparently associated with a parallel increase in tubular secretion of HCQ enantiomers in diabetic rats. Our results indicate that diabetes causes a significant increase in non-renal clearance of both HCQ enantiomers. However, the extent of increase was more pronounced for S-HCQ than R-HCQ. Significant decrease in elimination $t_{1/2}$ of the enantiomers may stem from the fact that an increase in the systemic clearance of the enantiomers was not associated with an increase in volume of distribution.

Since early kidney hyperfiltration and altered cytochrome P-450 metabolizing activity are documented in human diabetes, a similar effect should be expected in diabetic patients. Further studies on the pharmacokinetics of HCQ in diabetic patients are, therefore, warranted.

Inflammation in adjuvant-induced arthritic rats resulted in significant decreases in total body clearance and volume of distribution for both HCQ enantiomers. The decreased volume of distribution in arthritic rats indicates that lowered clearance could, in part, be due to an overall change in protein and platelet binding. This could be further supported by a significant decrease in the free fraction of HCQ enantiomers in platelet-rich plasma of arthritic rats as compared to that of healthy animals. Increased blood concentrations of the HCQ enantiomers were found to be significantly related to the degree of inflammation. This indicates that a strong relationship exists between the reduced clearance associated with arthritis and the severity of the disease. Similar to our observations of HCQ disposition in arthritic rats, higher blood concentrations of both R- and S-HCQ may also occur in human arthritic subjects due to increased binding and reduction in organ function.

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